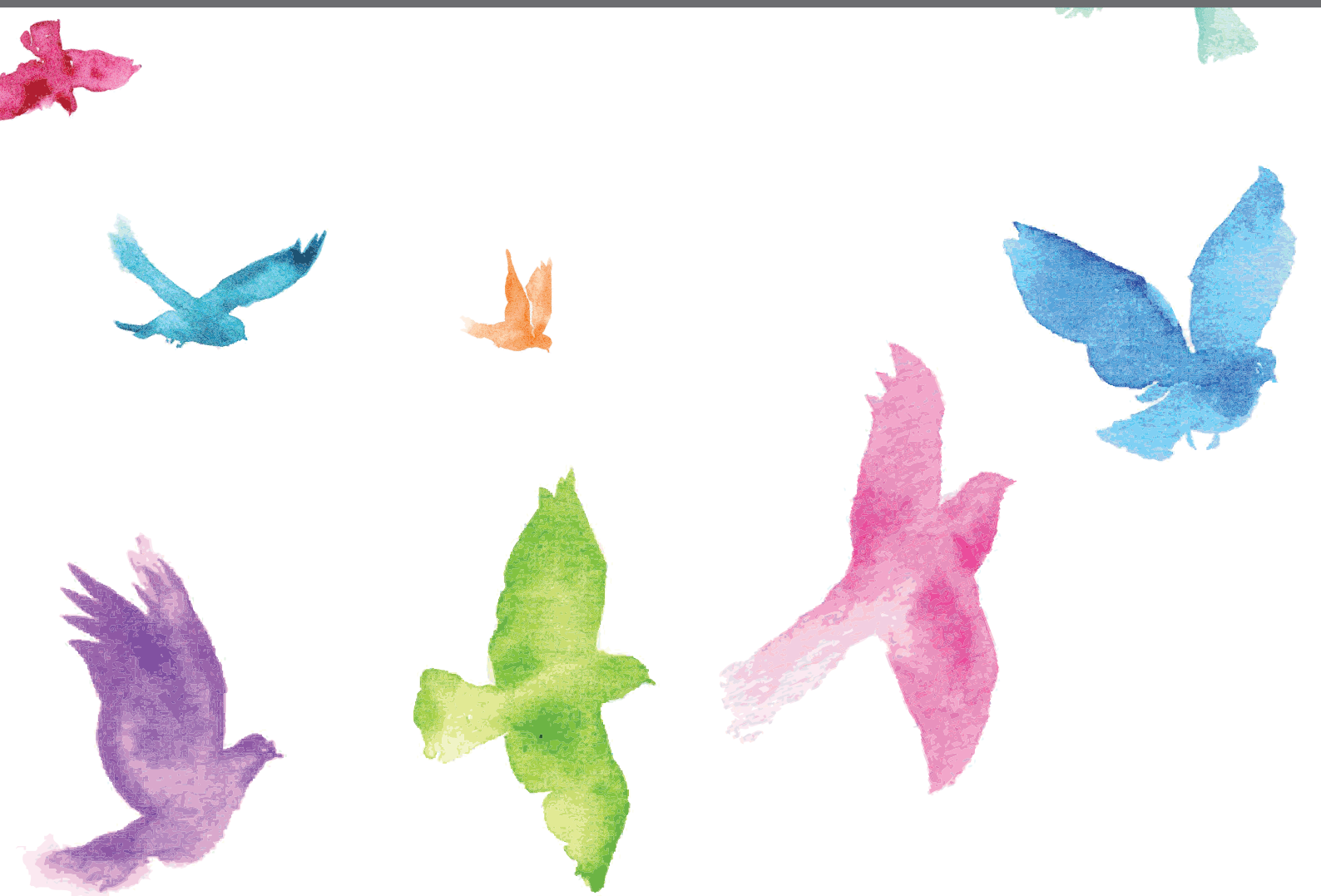
NON-TARGET EFFECTS OF PESTICIDES ON ORGANISMS INHABITING AGROECOSYSTEMS

EDITED BY: Johann G. Zaller and Carsten A. Brühl
PUBLISHED IN: Frontiers in Ecology and Evolution



frontiers Research Topics

Frontiers Copyright Statement

© Copyright 2007-2019 Frontiers Media SA. All rights reserved.

All content included on this site, such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of or is licensed to Frontiers Media SA ("Frontiers") or its licensees and/or subcontractors. The copyright in the text of individual articles is the property of their respective authors, subject to a license granted to Frontiers.

The compilation of articles constituting this e-book, wherever published, as well as the compilation of all other content on this site, is the exclusive property of Frontiers. For the conditions for downloading and copying of e-books from Frontiers' website, please see the Terms for Website Use. If purchasing Frontiers e-books from other websites or sources, the conditions of the website concerned apply.

Images and graphics not forming part of user-contributed materials may not be downloaded or copied without permission.

Individual articles may be downloaded and reproduced in accordance with the principles of the CC-BY licence subject to any copyright or other notices. They may not be re-sold as an e-book.

As author or other contributor you grant a CC-BY licence to others to reproduce your articles, including any graphics and third-party materials supplied by you, in accordance with the Conditions for Website Use and subject to any copyright notices which you include in connection with your articles and materials.

All copyright, and all rights therein, are protected by national and international copyright laws.

The above represents a summary only. For the full conditions see the Conditions for Authors and the Conditions for Website Use.

ISSN 1664-8714
ISBN 978-2-88945-976-6
DOI 10.3389/978-2-88945-976-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

NON-TARGET EFFECTS OF PESTICIDES ON ORGANISMS INHABITING AGROECOSYSTEMS

Topic Editors:
Johann G. Zaller, University of Natural Resources and Life Sciences Vienna (BOKU), Austria
Carsten A. Brühl, University of Koblenz-Landau, Germany

Pesticide usage is increasing worldwide and considered among the main factors contributing to the global decline in biodiversity. This Research Topic provides an overview of the state-of-knowledge regarding non-target effects of herbicides, fungicides, insecticides and rodenticides on a variety of ecosystem functions and organisms. Taxa covered in the contributions include algae, amphibians, aquatic fungi, aquatic insects, bats, bumblebees, butterflies, earthworms, enchytraeids, honeybees, plants, rodents and soil microorganisms. The papers also highlight many gaps in our understanding of non-target effects of pesticides and their consequences for biodiversity and functions of various ecosystems. Overall, it became clear that priorities for future work on pesticides and their effects should more focus on investigating or simulating realistic field situations, i.e., multiple applications of pesticides during the growing season including their temporal and spatial interactions with fauna and flora.

Citation: Zaller, J. G., Brühl, C. A., eds. (2019). Non-Target Effects of Pesticides on Organisms Inhabiting Agroecosystems. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88945-976-6

Table of Contents

frontiers in Environmental Science

EDITORIAL
published: 31 May 2019
doi: 10.3389/fenvs.2019.00075

Editorial: Non-target Effects of Pesticides on Organisms Inhabiting Agroecosystems

Johann G. Zaller[1]* *and Carsten A. Brühl*[2]

[1] Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria, [2] Community Ecology and Ecotoxicology, Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany

Keywords: ecotoxicology, plant protection, agroecology, regulatory toxicity, agrochemicals

Editorial on the Research Topic

Non-target Effects of Pesticides on Organisms Inhabiting Agroecosystems

Pesticides are increasingly used worldwide and concurrently, evidence is mounting that they have detrimental ecological effects on the biodiversity of organisms in agricultural landscapes. However, pesticides are also perceived as a highly regulated group of chemicals in respect of their risk for the environment. The aim of this Research Topic, is to provide an overview of the state of knowledge regarding non-target effects of pesticides and to identify knowledge gaps. We collated in total 14 papers written by 61 authors, eight of which are original research papers and six are reviews. Many papers dealt with effects of herbicides and insecticides. Effects of glyphosate and neonicotinoids were addressed for various groups of organisms. But also fungicides as well as rodenticides were in the focus of researchers and some addressed more than one pesticide class. Several papers explicitly made recommendations for amendments to current risk assessment approaches required for the regulation of pesticides.

The mode-of-action of glyphosate-based herbicides is still not completely understood. Gomes et al. tested glyphosate-dependent inhibition of photosynthesis in willow. The authors showed that glyphosate promotes changes in the photosynthetic apparatus leading to decreased photochemistry. For the first time, interconnecting effects on shikimate pathway, photosynthetic process and oxidative events in plants were presented. This research documented that glyphosate induces a series of interconnected events that leads to decreased photosynthetic activity in willow plants and indicates how glyphosate tolerance in plants may develop through the activation of antioxidant systems.

Herbicide drift effects in field margin plant community have been little studied so far. In a field trial Nelemans et al. investigated effects of an herbicide (metsulfuron-methyl) on sown plant communities. The herbicide drift affected biomass production, plant cover and seed germination of several plant species. The study implies that spray drift leads to shifts in species compositions and succession in vegetation in off-crop areas adjacent to arable fields.

Herbicides can not only affect the off-field plants but also soil microorganisms as studied by Aristilde et al. Their results show that glyphosate altered metabolite levels in the biosynthetic pathway of aromatic amino acids in the soil bacterium Pseudomonas. The greatest inhibition was found for tryptophan, an important precursor to secondary metabolites. The authors conclude that the glyphosate-induced specific disruption of *de novo* biosynthesis of aromatic amino acids accompanied by widespread metabolic disruptions was responsible for the observed dose-dependent adverse effects. Their findings suggest that rhizospheric bacteria may be less susceptible to glyphosate effects due to the high-carbon environment in the rhizosphere with relatively higher concentration of amino acids and sugars relative to bulk soils.

OPEN ACCESS

Edited and reviewed by:
Peter Thorburn, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

***Correspondence:**
Johann G. Zaller
johann.zaller@boku.ac.at

Specialty section:
This article was submitted to Agroecology, a section of the journal Frontiers in Environmental Science

Received: *23 January 2019*
Accepted: *14 May 2019*
Published: *31 May 2019*

Citation:
Zaller JG and Brühl CA (2019) Editorial: Non-target Effects of Pesticides on Organisms Inhabiting Agroecosystems. Front. Environ. Sci. 7:75. doi: 10.3389/fenvs.2019.00075

It is rarely considered that amphibian species migrating through arable fields may be exposed to pesticides. Berger et al. evaluated regional migration patterns and glyphosate applications in German agriculture over 20 years. Their results reveal the highest increase in temporal coincidence with glyphosate application for both adult and juvenile Great crested newt (*Triturus cristatus*) and Fire-bellied toad (*Bombina bombina*). The authors demand that pesticide risk assessment should start considering potential amphibian exposure and include resulting effects in the regulatory procedure.

Interactive effects between glyphosate-based herbicides and environmental parameters were studied by Baier et al. Results of this laboratory experiment showed that eggs of Common toads (*Bufo bufo*) were more sensitive to herbicides than tadpoles. Interaction between herbicide concentrations and temperature resulted in more pronounced glyphosate effects at lower temperatures. This is remarkable as ecotoxicologial studies for regulatory risk assessment are not evaluating temperature as a co-stressor and therefore the actual risk might be underestimated.

In their contribution, Gomes and Juneau reviewed how variations in climatic conditions influence herbicide toxicity in algae and cyanobacteria. They show that responses to interactions between light, temperature, and herbicides are species-specific, making it difficult to establish a single model of how climate change will affect herbicide toxicity for aquatic algae. The authors also demand the inclusion of environmental parameters when assessing pesticide risk.

In his mini-review Stoner shows that current neonicotinoid risk assessment does not adequately address differences between honey bees (*Apis mellifera*) and bumble bees (*Bombus* spp.). Because bumble bee queens are solitary for some time and forage for pollen and nectar themselves, their pesticide exposure is higher than for honey bee queens, where worker bees provide food and are therefore potentially exposed to pesticides. Additional research focusing on critical periods in a bumble bee queen's life and the risk for pesticide exposure and related effects is demanded.

Neonicotinoids and their effects on butterflies, including also sublethal endpoints, were addressed in a systematic review by Mulé et al. The insecticides cause negative effects such as reduced survival rate, feeding interruption, and alteration of oviposition behavior. The paper highlights that it has been impossible to determine which butterfly species is the most sensitive so far.

The impact of insecticides on aquatic environments was reviewed by Sánchez-Bayo et al. The paper shows a widespread aquatic contamination by neonicotinoids and identifies the communities most at risk. Gaps in knowledge stem from difficulties in obtaining long-term experimental data relating effects on individual organisms to impacts on populations and ecosystems.

Single and combined effects of insecticide and fungicide seed dressings and subsequent herbicide application on soil biota and processes were studied by van Hoesel et al. Seed dressings in winter wheat significantly reduced the surface activity of earthworms with no difference whether insecticides or fungicides were used. The authors conclude that interactive effects on soil biota and processes of different systemic pesticide classes should receive more attention.

Although agriculture dominates much of Europe's landscape, there is no information on bat activity in different crops and therefore exposure cannot be addressed appropriately. Stahlschmidt et al. investigated foraging activity of bats in an agricultural landscape in Germany. In 300 accumulated sampling nights a total of 14 bat species were recorded. The high bat activity levels above crops, related pesticide inputs and the availability of prey insects, makes a dietary exposure of bats likely. Bats and their exposure toward pesticides are currently not considered in the EU pesticide regulation.

An often-ignored aspect was addressed by Topping and Elmeros: The exposure of mammalian predators to anticoagulant rodenticides was investigated using a spatio-temporal model supported by an experimental study. The paper suggests that the driver of high anticoagulant rodenticides incidence in non-target small mammal predators is likely to be related to use patterns.

In their review Römbke et al. summarized ecotoxicological effects of pesticides on enchytraeids (Oligochaeta) in agroecosystems. Because of their close contact with the soil pore water, a high ingestion rate and a thin cuticle, they show a high sensitivity to a broad range of pesticides. The authors recommend the use of enchytraeids in pesticide risk assessments because of their diversity, functional importance and simple use in standardized tests.

In their review, Ittner et al. draw attention to aquatic fungi as a disregarded trophic level, not addressed in regulatory aquatic risk assessment so far. Freshwater fungi are a diverse group and fulfill important functions in the food web dynamics of surface water ecosystems. The authors conclude that development and standardization of different fungi bioassays is needed to effectively protect food-webs in aquatic ecosystems.

Taken collectively, we are grateful to all contributors for presenting such a variety of aspects at different complexity levels from physiology to ecosystem functioning. It also became clear that we deploy enormous amounts of pesticides that reach different compartments of the ecosystems in the agricultural landscape with little knowledge on their non-target effects. As a consequence, dramatic declines in biodiversity in insects, birds and other organisms have been related to pesticide pollution (Geiger et al., 2010; Beketov et al., 2013; Sánchez-Bayo and Wyckhuys, 2019). This should alert us to radically challenge and completely remodel the current procedures for environmental risk assessments of pesticides as they are obviously inadequate to protect the biodiversity and integrity of ecosystems. All contributions from this Research Topic also highlighted a great array of remaining knowledge gaps, among them are (not exhaustive):

- effects of additives and surfactants in pesticide formulations rather than active ingredients
- effects on realistic field populations and communities rather than singe species studies
- effects of more than one pesticides resulting from tank mixtures and real-life application sequences
- carry-over and legacy effects

- effects in agricultural fields and neighboring ecosystems (e.g., pesticide drift)
- interactions with environmental stressors such as organismic competition, abiotic factors (temperature, moisture), soil types (humus content), and climate change

In conclusion, priorities for future work on pesticides and their effects should focus on investigating or simulating realistic field situations, i.e., multiple applications of pesticides during the growing season including their temporal and spatial interactions with fauna and flora.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Beketov, M. A., Kefford, B. J., Schäfer, R. B., and Liess, M. (2013). Pesticides reduce regional biodiversity of stream invertebrates. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11039–11043. doi: 10.1073/pnas.1305618110

Geiger, F., Bengtsson, J., Berendse, F., Weisser, W. W., Emmerson, M., and Morales, M. B., (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic Appl. Ecol.* 11, 97-105. doi: 10.1016/j.baae.2009.12.001

Sánchez-Bayo, F., and Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: a review of its drivers. *Biol. Conserv.* 232, 8–27. doi: 10.1016/j.biocon.2019.01.020

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Zaller and Brühl. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 February 2017
doi: 10.3389/fpls.2017.00207

Glyphosate-Dependent Inhibition of Photosynthesis in Willow

Marcelo P. Gomes[1,2], Sarah G. Le Manac'h[1], Louise Hénault-Ethier[3], Michel Labrecque[4], Marc Lucotte[3] and Philippe Juneau[1,3]**

[1] Ecotoxicology of Aquatic Microorganisms Laboratory, GRIL, TOXEN, Department of Biological Sciences, Université du Québec à Montréal, Montréal, QC, Canada, [2] Laboratório de Fisiologia Vegetal, Instituto de Ciências Biológicas, Departamento de Botânica, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [3] Institut des Sciences de l'Environnement, Université du Québec à Montréal, Montréal, QC, Canada, [4] Institut de Recherche en Biologie Végétale, Montreal Botanical Garden, Montréal, QC, Canada

We studied the physiological mechanisms involved in the deleterious effects of a glyphosate-based herbicide (Factor® 540) on photosynthesis and related physiological processes of willow (*Salix miyabeana* cultivar SX64) plants. Sixty-day-old plants grown under greenhouse conditions were sprayed with different rates (0, 1.4, 2.1, and 2.8 kg a.e ha^{-1}) of the commercial glyphosate formulated salt Factor® 540. Evaluations were performed at 0, 6, 24, 48, and 72 h after herbicide exposure. We established that the herbicide decreases chlorophyll, carotenoid and plastoquinone contents, and promotes changes in the photosynthetic apparatus leading to decreased photochemistry which results in hydrogen peroxide (H_2O_2) accumulation. H_2O_2 accumulation triggers proline production which can be associated with oxidative protection, $NADP^+$ recovery and shikimate pathway stimulation. Ascorbate peroxidase and glutathione peroxidase appeared to be the main peroxidases involved in the H_2O_2 scavenging. In addition to promoting decreases of the activity of the antioxidant enzymes, the herbicide induced decreases in ascorbate pool. For the first time, a glyphosate-based herbicide mode of action interconnecting its effects on shikimate pathway, photosynthetic process and oxidative events in plants were presented.

Keywords: herbicide, oxidative stress, photosynthesis, proline, shikimate, willow

OPEN ACCESS

Edited by:
Johann G. Zaller, University of Natural Resources and Life Sciences, Vienna, Austria

Reviewed by:
Christine Helen Foyer, University of Leeds, UK
Autar Krishen Mattoo, United States Department of Agriculture, USA

***Correspondence:**
Marcelo P. Gomes
marcelopgom@yahoo.com.br
Philippe Juneau
juneau.philippe@uqam.ca

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Plant Science

Received: *22 November 2016*
Accepted: *03 February 2017*
Published: *17 February 2017*

Citation:
Gomes MP, Le Manac'h SG, Hénault-Ethier L, Labrecque M, Lucotte M and Juneau P (2017) Glyphosate-Dependent Inhibition of Photosynthesis in Willow. Front. Plant Sci. 8:207. doi: 10.3389/fpls.2017.00207

INTRODUCTION

Glyphosate [*N*-(phosphonomethyl)glycine)] is the most broadly used herbicide worldwide since the introduction of glyphosate-resistant (GR) plants (Coupe et al., 2012). Although it has been suggested as one of the least toxic pesticides to animals and humans (Williams et al., 2000; Cerdeira and Duke, 2006), the widespread use of glyphosate together with its great solubility trigger some concerns about its possible effects on the environment.

Glyphosate negative effects on non-target plants (Bott et al., 2011) and aquatic organisms (Vendrell et al., 2009; Inderjit and Kaushik, 2010) have been largely described. By inhibiting the

Abbreviations: APX, ascorbate peroxidase; AsA, reduced ascorbate; CAT, catalase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; DHA, dehydroascorbate – oxidized ascorbate; ETR_{max}, maximum electron transport rate; F_V/F_M, maximal photochemical efficiency of PSII; GPX, glutathione peroxidase; GR, glutathione reductase; g_s, stomatal conductance; H_2O_2, hydrogen peroxide; I_k, minimum saturating irradiance; MDA, malondialdehyde – lipid peroxidation; NPQ, non-photochemical quenching; PQ, plastoquinone; PS, photosystem; qP, photochemical quenching; RLC, rapid light curve; ROS, reactive oxygen species; SOD, superoxide dismutase; UQF_{rel}, relative unquenched fluorescence.

EPSP synthase (EC 2.5.1.19), glyphosate-based herbicides prevent biosynthesis of aromatic amino acids (Siehl, 1997) leading to shikimic acid accumulation (Duke and Powles, 2008). The depletion of the aromatic amino acid pool leads to a reduction of protein synthesis necessary to growth maintenance (Siehl, 1997). On the other hand, in some plants, aromatic amino acid deficiencies upon glyphosate application has not been found, although deleterious effects of the herbicide have been observed (Lee, 1981; Wang, 2001; Serra et al., 2013). This indicates that glyphosate can affect other plant-physiological processes (Gomes et al., 2014b). Numerous studies demonstrated decreases in the photosynthetic rate of plants following treatment with glyphosate (Mateos-Naranjo et al., 2009; Yanniccari et al., 2012; Zobiole et al., 2012). In glyphosate-sensitive plants, the herbicide causes inhibition of CO_2 assimilation (Diaz Vivancos et al., 2011) and depletion of intermediates of the photosynthetic carbon reduction cycle (Servaites et al., 1987) which could be linked to the unregulated flux of carbon into the shikimate pathway (Siehl, 1997). Moreover, glyphosate can indirectly affect photosynthesis by inhibiting chlorophyll biosynthesis (Fedtke and Duke, 2005) or inducing chlorophyll degradation (Gomes et al., 2016a), decreasing stomatal conductance (Yanniccari et al., 2012), and provoking nutritional disturbances (Cakmak et al., 2009; Su et al., 2009). Nowadays, a special attention has been giving to understand glyphosate-induced oxidative stress in plants (Gomes et al., 2014b).

Reactive oxygen species are essential in plant signaling; however, once accumulated, ROS become toxic, inducing irreversible changes in metabolism, cell cycle, and increase oxidative bursts (Gomes et al., 2014a). By interacting with biological molecules, ROS can induce destruction of DNA, lipids, and proteins (Foyer and Noctor, 2011). To avoid oxidative damage due to ROS accumulation, plants have developed enzymatic (e.g., SOD, CAT, APX, GPX, and GR) and non-enzymatic (e.g., ascorbate and glutathione) systems (Foyer and Noctor, 2011). The activity of antioxidant systems as well as the lipid peroxidation extent are oxidative stress markers which were shown to be modulated by glyphosate exposure (Ahsan et al., 2008; Moldes et al., 2008; Miteva et al., 2010).

Glyphosate effects on photosynthesis of non-resistant plants were associated to the herbicide induced decreases in the abundance of photosynthetic pathway proteins together with the oxidation of the major redox pools (Diaz Vivancos et al., 2011). However, it has been reported that glyphosate can also induce ROS accumulation (Ahsan et al., 2008; Moldes et al., 2008; Miteva et al., 2010; Gomes et al., 2016a) and glyphosate-resistance was related to the ability of plants to avoid oxidative bursts through activation of antioxidant systems (Maroli et al., 2015). Photosynthesis-targeting herbicides, such as atrazine, are known to induce oxidative stress by inhibiting Hill's reactions (Fedtke and Duke, 2005). Plants exposed to these kinds of herbicides are not able to cope with the mass of triplet chlorophyll molecules produced due the blockage of the electron transport flow, resulting in cell oxidative bursts due to ROS accumulation. On the other hand, it is not clear how glyphosate can induce ROS accumulation in plants and if the oxidative stress induced by the herbicide could also be related to the observed decreases in photosynthesis. We hypothesized that the interference on shikimate pathway could induce ROS production and consequently affect photosynthesis of exposed plants. Therefore, in this study we accessed the physiological mechanisms involved in the deleterious effects of a glyphosate-based herbicide (Factor® 540) on photosynthesis of willow (*Salix miyabeana* cultivar SX64) plants. For the first time, a glyphosate-based herbicide mode of action interconnecting glyphosate effects on shikimate pathway, plant photosynthetic process and oxidative events were described.

MATERIALS AND METHODS

Greenhouse Experiments

Salix miyabeana cultivar SX64 was chosen for this study due to its high tolerance to stress factors, fast growth and great biomass production (Labrecque and Teodorescu, 2005). Moreover, this species has been indicated for phytoremediation programs, particularly in the context of riparian buffer strips, to reclaim agricultural contaminants (Gomes et al., 2016b). Cuttings of *S. miyabeana* approximately 20 cm long were grown in plastic boxes (35 l) filled with distilled water amended with King Max nutrient solutions A (7% P_2O_5, 11% K_2O, 1.5% Mg, 1.27% S, 0.07% B, 0.002% Mo, 0.12% Zn) and B (4% N, 1% NH_4^+, 3% NO_3^{-2}, 10% K_2O, 2% Ca, 0.05% Fe, 0.05% Mn) (Montreal, QC, Canada), following the product's instructions. The solutions were continuously aerated, and renewed every 15 days. The pH of the medium was checked and adjusted on a weekly basis to 6.5 ± 0.1. The greenhouse was maintained at 25/22°C (±3°C) day/night temperature with natural light supplemented by sodium vapor lamps to provide a 12 h photoperiod and an average photosynthetic active radiation of 825 μmol photons m^{-2} s^{-1}. After an initial growth period (45 days), rooted, healthy (without leaf chlorotic spots) and uniform (similar height) plants were used in all treatments. A randomized block design with seven containers (corresponding to the replicates) per treatment, in a 4 (herbicide concentrations) × 4 (times of evaluation) factorial scheme was used. One hundred microliters of a freshly prepared herbicide solutions were hand-sprayed uniformly on each of the first three fully expanded leaves (corresponding to seventh to ninth leaves counting down from the shoot apex). This spray volume did not result in any runoff from the leaves. The herbicide (0, 56.15, 84.21, and 112.30 mM of glyphosate) applied concentrations were equivalent to field applications of 0, 1.4, 2.1, and 2.8 kg glyphosate ha^{-1}, which represent scenarios of 50, 75, and 100% of the standard field herbicide concentration applied in agricultural areas in Quebec (Gomes et al., 2016a).

Photosynthetic (using chlorophyll fluorescence kinetic measurements) and biochemical evaluations were performed at 0, 6, 24, 48, and 72 h after the beginning of the treatments. The evaluations were stopped after 72 h of exposure as plants from the highest glyphosate treatment showed pronounced intoxication symptoms, including several necrotic spots and loss of leaves (data not shown). After photosynthetic and stomatal conductance evaluations, plants were harvested and thoroughly washed with distilled water. Samples of the seventh (first fully

expanded leaf from the apex) to ninth leaves were immediately frozen in liquid nitrogen and stored in aluminum foil paper at −80°C until biochemical evaluations and oxidative damage evaluations.

Gas Exchange, Chlorophyll Fluorescence, and Pigment Concentrations

Gas exchange, chlorophyll fluorescence, and pigment contents were measured on samples from the first, second, and third fully expanded leaves (seventh–ninth leaves from the apex), which also received the herbicide, for a total of three measurements per plant. Measurements of stomatal conductance (g_s) were performed using a leaf porometer (model SC-1, Decadon Devices Inc., Washington, DC, USA). Then, these leaves were dark-acclimated for 20 min and the chlorophyll fluorescence emission was assessed using a pulse-amplitude modulation (PAM) fluorometer (model PAM-2500, WALZ, Effeltrich, Germany). A RLC analysis was performed according to Juneau et al. (2015). An 11 steps RLC was performed. Saturating pulses were triggered at 0.8 min intervals with varying actinic light intensity for each step (0, 31, 48, 76, 117, 179, 253, 405, 586, 874, and 1326 μmol photons m^{-2} s^{-1}). Using the RLC, the evaluation of the following parameters was performed: the ETR (Krall and Edwards, 1992), the qP (van Kooten and Snel, 1990), the UQF_{rel} (Juneau et al., 2005), the NPQ (Redondo-Gómez et al., 2008), and the F_V/F_M (Kitajima and Butler, 1975). To compare treatments, fluorescence results from the 874 μmol photons m^{-2} s^{-1} (most similar irradiation in relation to light growth conditions) were used. Curves of ETR versus irradiance were also plotted and the ETR_{max} and the I_k were calculated according to Eilers and Peeters (1988).

For pigments evaluations, three foliar disks of approximately 5 mm in diameter were taken from each leaf, and after determining the fresh weight of the samples, their chlorophyll and carotenoid pigments were extracted in 80% acetone after macerating the disks with a mortar and pestle. The spectral absorption of the extracts (from 300 to 800 nm) was measured using a Varian Cary® 300 Bio UV-Vis spectrophotometer (Varian, USA). The concentrations (μg/g fresh leaf weight) of total chlorophylls and carotenoids were calculated using the equations described by Lichtenthaler and Wellburn (1983).

Biochemical Evaluations

Shikimate and proline concentrations were evaluated following the methods described in Bates et al. (1973) and Bijay and Dale (1998), respectively. To evaluate the pool of quinones in leaves, 0.1 g of fresh plant tissue was ground in liquid nitrogen, homogenized in 1000 μl of freeze-cold ethyl acetate and then centrifuged for 1 min at 6.590 × *g* (Kruk and Karpinski, 2006). The supernatant was then transferred to a collecting tube and the procedure was repeated twice (by adding 1000 μl of freeze-cold ethyl acetate to the pellet) to assure high extraction efficiency. Ten microliters of cold 1 M sodium borohydride ($NaBH_4$) was added to the combined supernatant to convert quinone to its reduced form and then, samples were centrifuged for 2 min at 10.000 × *g* to remove impurities (Yoshida et al., 2010). The standard of plastoquinone (PQ-9, 1 mM) was acquired from the laboratory of J. Kruk (Jagiellonian University, Poland). After dilution in ethanol, the amount of 20 μl of cold 1 M $NaBH_4$ was added to assure complete reduction of plastoquinone pool. The UHPLC (Agilent 1290 Infinity II LC, Wilmington, DE, USA) measurements were performed according to Yoshida et al. (2010), using UV-VIS detector, fluorescence detector, column (50 mm × 2.1 mm) isocratic solvent system (methanol/hexane, 340/20 vol/vol), flow rate of 0.31 ml/min, absorption detection wavelength at 255 nm, fluorescence excitation/emission detection at 290/330 nm, and injection volume of 1 μl.

To assess oxidative responses, H_2O_2, MDA contents and the activity of antioxidant systems were studied following the methods described by Gomes et al. (2014c). H_2O_2 was extracted in 2 ml of 0.1% trichloroacetic acid (TCA) and after centrifugation at 12000 × *g* for 15 min, 300 μl of the centrifuged supernatant was reacted with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. Samples were read at 390 nm and H_2O_2 concentrations were determined using an extinction coefficient (ε) of 0.28 mM^{-1} cm^{-1}. The estimation of lipid peroxidation was based on the production of 2-thiobarbituric acid reactive metabolites, particularly MDA. Samples containing 200 mg of leaf and root tissue were macerated in 5 mL of 0.1% TCA. After complete homogenization, 1.4 mL of the homogenate was transferred to an eppendorf tube and centrifuged at 10,000 rpm for 5 min. An aliquot of 0.5 mL of the supernatant was added to 2 mL 0.5% (v/v) TBA (thiobarbituric acid) in 20% TCA. The mixture was heated in a water bath at 95°C for 30 min and then ice-cooled for 10 min. Readings were taken using a spectrophotometer at 535 and 600 nm.

To study the antioxidant enzymes, 0.1 g of leaves were macerated in 800 μl of an extraction buffer containing 100 mM potassium buffer (pH 7.8), 100 mM EDTA, 1 mM L-ascorbic acid and 2% PVP (m/v). The protein contents of samples were determined using the Bradford method. Activities of SOD (EC 1.15.1.1), CAT (EC1.11.1.6), APX (EC 1.11.1.11), GPX (E.C. 1.11.1.9), and GR (E.C. 1.6.4.2) were assessed. To evaluate the ascorbate pool [total ascorbate (AsA + DHA), AsA and DHA], 0.2 g of frozen tissue were ground in liquid nitrogen in a mortar and pestle and homogenized with 5 ml of 6.5% (w/v) *m*-phosphoric acid containing 1 mM NaEDTA.

Statistical Analyses

Results were expressed as the average of three replicates. Statistical analyses were performed using JMP software 10.0 (SAS Institute Inc). Results were submitted to normality (Shapiro–Wilk) and homogeneity (Bartlett) tests and then statistically evaluated. MANOVA univariate repeated measures, with Time as the within-subject factor and the herbicide concentrations as the main effects, were used to analyze differences in the variables studied during exposure to the treatments. Glyphosate, Time, and the interaction between glyphosate and time were included within the model. The sphericity of the data was tested by the Mauchly's criteria to determine whether the univariate *F* tests for the within-subject effects were valid. In cases of invalid *F*, the Greenhouse–Geisser test was used to estimate epsilon

(ε). Contrast analysis was used when there were significant differences in the variables between treatments (Supplementary Tables 1S and 2S).

RESULTS

Pigment Content, Gas Exchange, and Chlorophyll Fluorescence

Total chlorophyll and plastoquinone concentrations were decreased in leaves of plants by herbicide exposure and by treatment time ($P > 0.001$; **Figure 1**). The carotenoid concentration was greater in herbicide-treated plants at 6 h for all applied doses (**Figure 1**); then, carotenoid concentration was decreased in plants exposed for at least 24 h to herbicide concentrations ($P < 0.0001$). The stomatal conductance was decreased in herbicide-exposed plants for all the treatment times ($P < 0.05$; **Figure 1**). Similar effects were observed on the ETR_{max}, the I_k, and the qP, which were significantly reduced in treated plants ($P < 0.0001$; **Figure 2**). However, for the first evaluation (6 h), ETR_{max}, I_k, and qP were not decreased in plants treated with 1.4 kg a.e ha^{-1} ($P > 0.05$; **Figure 2**). The UQF_{rel} increased in all treated plants (**Figure 1**). Concomitantly, the NPQ decreased in plants exposed for more than 24 h to the herbicide ($P < 0.05$; **Figure 2**). The maximal PSII photochemical efficiency (F_V/F_M) was decreased in herbicide-treated plants ($P < 0.0001$). Decreased F_V/F_M was seen in plants treated with 1.4 kg a.e ha^{-1} only after 72 h of herbicide exposure ($P < 0.05$; **Figure 2**). Plants exposed to 2.1 kg a.e ha^{-1} showed decreases in F_V/F_M at 48 and 72 h of exposure ($P < 0.001$). In contrast, in all the evaluations, plants exposed to 2.8 kg a.e ha^{-1} showed decreased F_V/F_M ($P < 0.01$; **Figure 2**).

Shikimate and Proline Contents

The shikimate and proline concentrations in leaves of herbicide-treated plants were always higher than the control ($P < 0.0001$; **Figure 3**). In plants exposed to 2.1 and 2.8 kg a.e ha^{-1}, an important shikimate accumulation was found after 72 h of herbicide-treatment ($P < 0.0001$).

H_2O_2 Contents and Lipid Peroxidation

Compared to control, H_2O_2 concentration was always higher in plants exposed to the herbicide ($P < 0.001$; **Figure 3**), and greatly increased in these plants after 72 h ($P < 0.01$). Similarly, lipid peroxidation (MDA concentration) was always higher in plants exposed to the herbicide (Supplementary Table 2S; **Figure 3**). In all plants, MDA content slightly increased at 24 h ($P > 0.05$). However, in plants treated with herbicide, a pronounced increase in MDA concentration was observed at 72 h ($P < 0.05$).

Antioxidant Responses

Plants treated with herbicide showed higher activity of all evaluated antioxidant enzymes after 6 h in relation to control ($P < 0.05$; **Figure 4**). We found that: (1) SOD and APX activities were higher in herbicide-treated plants up to 24 h ($P < 0.0001$), and then were reduced for the following exposure times ($P < 0.0001$); (2) CAT activity was always higher in plants treated with herbicide ($P < 0.0001$); (3) similar to SOD and APX, GPX activity was also reduced in herbicide treated plants at 48 and 72 h of exposure ($P < 0.0001$); (4) GR activity was higher in herbicide treated plants up to 48 h of exposure ($P < 0.05$). Regarding ascorbate pool (**Figure 5**) we found that, in relation to control: (1) total ascorbate concentrations (AsA + DHA) were higher in herbicide-treated plants up to 24 h of exposure, and then were reduced for the following exposure times ($P < 0.0001$); (2) the concentrations of the ascorbate reduced form (AsA) were greater in control plants up to 24 h, did not differ between treatments at 48 h and was increased in herbicide treated plants for 72 h ($P < 0.0001$); (3) the concentrations of oxidized form of ascorbate (DHA) were greater in herbicide treated plants up to 24 h and were reduced for the following exposure duration ($P < 0.0001$); (4) the AsA/DHA ratio was lower in 6 and 24 h treated plants compared to control, but was higher for the following treatment times ($P < 0.0001$).

DISCUSSION

In this study, for the first time, a wide investigation of the impacts of glyphosate-based herbicide on several physiological processes was done. We demonstrated that this type of herbicide affected not only the shikimate pathway, but several physiological processes in willow plants as previously reported by Gomes et al. (2016b). **Figure 6** represents an integrative model interconnecting the studied physiological parameters (in particular, shikimate pathway, photosynthetic processes and oxidative events) affected by exposure to a glyphosate-based herbicide greater than 24 h (48 and 72 h). The various steps of this model are identified throughout the text as **Figure 6**, #1–19.

The glyphosate-based herbicide clearly inhibited the shikimate pathway in willow plants, as demonstrated by the shikimate accumulation (**Figure 3**) and also reported by Huang et al. (2012) and Gomes et al. (2016b). By inhibiting the shikimate pathway (**Figure 6**, #1), the glyphosate-based herbicide may prevent the biosynthesis of several secondary plant compounds (Siehl, 1997), including plastoquinones (**Figure 1**; **Figure 6**, #2). It is known that UQF_{rel} is an indicator of closed PSII reaction centers (RCs) present under continuous illumination (Juneau et al., 2005) and qP represents the proportion of open PSII RCs (Maxwell and Johnson, 2000). Therefore, the higher UQF_{rel} and lower qP in treated plants indicate that the plastoquinone pool, and thus the PSII RCs, were in a more reduced state than in control plants, a consequence of a lower PQ content (**Figure 6**, #2 and 3) and/or less effective PSI. This may, together with the decrease in the I_k (for doses $\geq$1.4 kg a.e ha^{-1}), have contributed to the observed lower ETR in treated plants (**Figure 2**; **Figure 6**, #4). Indeed, a lower ability for PSII to deliver electrons to the electron transport chain, leading to PSII saturation at low irradiance, may explain the observed decrease in photosynthesis observed here and in previous studies (Huang et al., 2012; Yanniccari et al., 2012; Gomes et al., 2016b). However, we demonstrated that other effects of the glyphosate-based herbicide have also caused the decrease in the ETR (see below).

The observed increase in the carotenoid concentration after 6 h in the herbicide-treated plants (**Figure 1**) could be related to the concomitant increase in H_2O_2 concentration, since it is known that ROS presence can induce carotenogenic responses (Fan et al., 1998). Indeed, by the activation of latent biosynthetic enzymes (such as glutathione transferase and glutathione reductase) or by the expression of genes coding for carotenogenic enzymes, ROS may regulate carotenoid concentration (Aniya and Anders, 1992; Bouvier et al., 1998). Since the maximal PSII photochemical yield (F_V/F_M) is a proxy of the PSII integrity (Walter et al., 2003), F_V/F_M up to 24 h in treated plants (with the exception of the highest dose; **Figure 2**) indicates that the glyphosate-based herbicide had no effect on the PSII integrity up to 24 h of exposure. Similarly, negative effects of glyphosate in F_V/F_M of *Lolium perenne* plants were only observed after 3 days of exposure (Yanniccari et al., 2012). This may be the consequence of the increased carotenoid concentration helping to prevent ROS-damages to PSII (Gomes et al., 2013b). Carotenoids are usually involved in the protection of the oxidative damage by the detoxification of oxygen singlets ($^1O_2$) produced by photosynthesis or by enzymatic conversion of other ROS to oxygen singlets (Boussiba, 2000). Although plants exposed to the highest herbicide doses contain high carotenoid concentration, this was not sufficient to prevent oxidative damages to PSII (since we observed lower F_V/F_M value). These plants also showed higher lipid peroxidation (**Figure 3**) indicating oxidative damages (Gunes et al., 2007), as it was also shown in maize (Sergiev et al., 2006) and rice (Ahsan et al., 2008). However, for exposure times longer than 24 h, plants treated with the glyphosate-based herbicide showed reduced carotenoid concentration (**Figure 1**). This can be a consequence of the inhibition of the shikimate pathway leading to decreased PQ concentration, since plastoquinone is a co-factor of the phytoene desaturase and ζ-carotene desaturase, enzymes involved in the carotenoid biosynthesis pathway (Sandmann et al., 2006). Therefore, decreased plastoquinone concentration will affect directly carotenoid biosynthesis (**Figure 6**, #5). In addition, the decrease in the non-photochemical energy dissipation (NPQ) [one of the mechanisms by which plants can dissipate excess light energy absorbed by PSII light-harvesting complexes in order to minimize the generation of the highly reactive $^1O_2$ responsible for oxidative damages (Demming-Adams and Adams, 2000)], in plants treated with the glyphosate-based herbicide (**Figure 2**) can be related to the decreased biosynthesis of carotenoids. β-carotene is known to be the precursor of zeaxanthin, the first compound of xanthophyll cycle (Bouvier et al., 1996), and therefore, reduced carotenoid concentration could lead to a lower efficiency of the xanthophyll cycle, reducing plant capacity for photoprotection and thus, leading to increased PSII damages (as shown by reduced F_V/F_M) (**Figure 6**, #6). These decreases in the

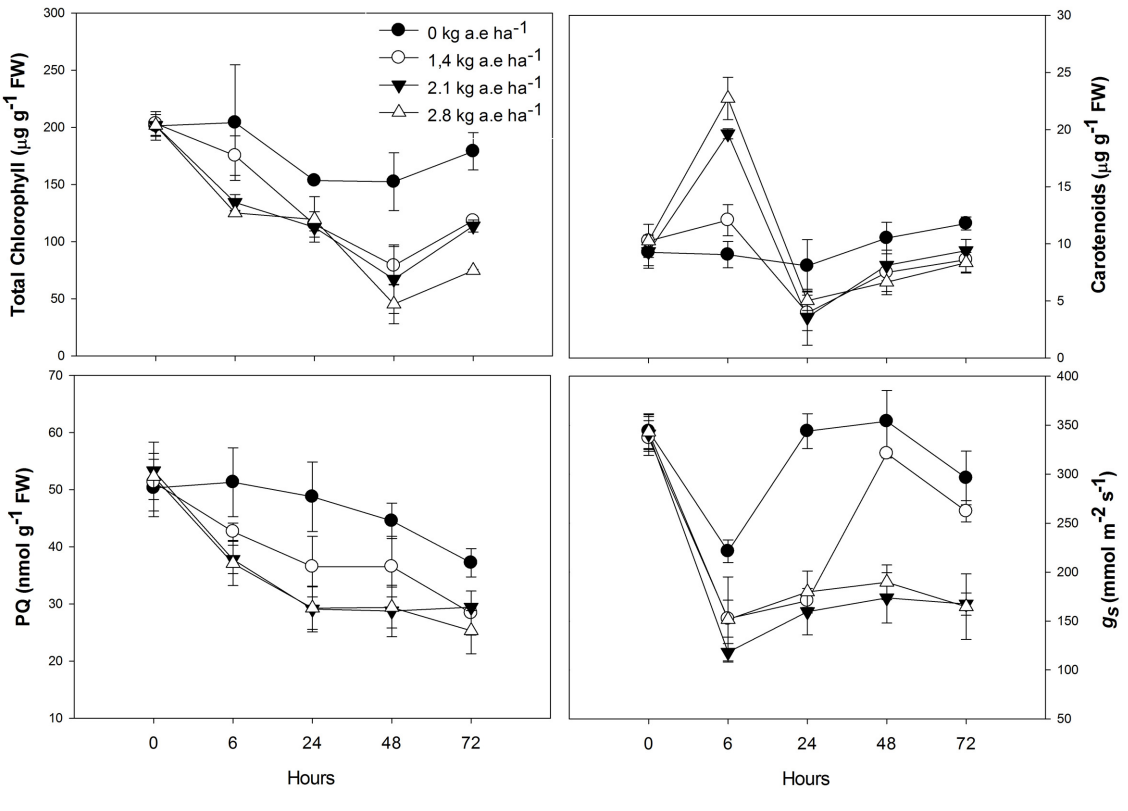


FIGURE 1 | Time courses of pigment (total chlorophyl and carotenoids) concentrations, plastoquinone pool (PQ), and stomatal conductance (g_s) in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of increased (0, 1.4, 2.1, and 2.8 kg a.e ha^{-1}) rates of the glyphosate based herbicide (Factor® 540). Values are means ± SE of three replicates.

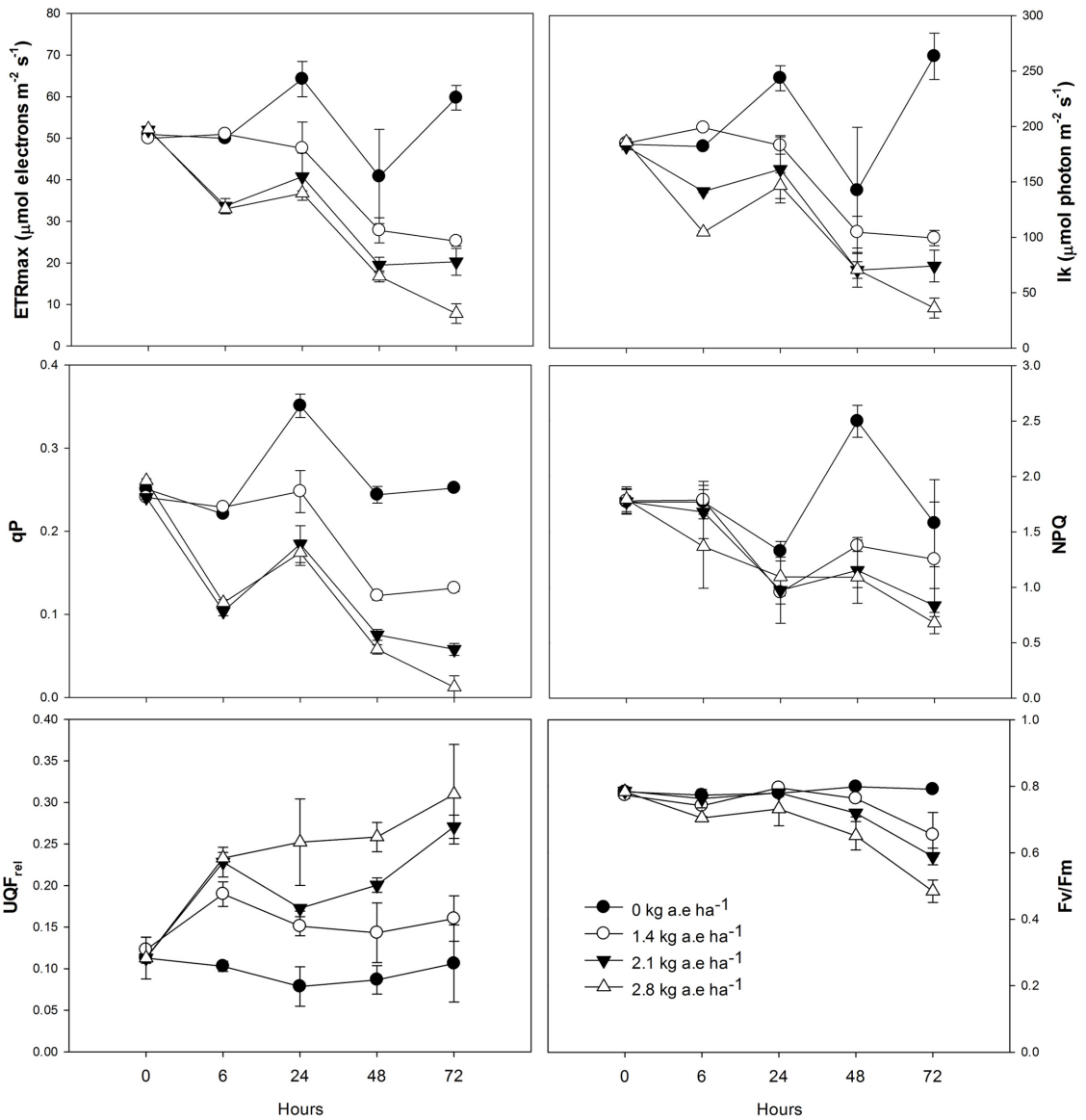


FIGURE 2 | Time courses of photosynthesis-related measurements [maximum electron transport rate (ETR_{max}), minimum saturating irradiance (I_k), photochemical quenching (qP), non-photochemical quenching (NPQ), relative unquenched fluorescence (UQF_{rel}), and maximal photochemical efficiency of PSII (F_V/F_M)] in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of increased (0, 1.4, 2.1, and 2.8 kg a.e ha^{-1}) rates of the glyphosate based herbicide (Factor® 540). Values are means ± SE of three replicates.

photosynthetic activity (shown by the decrease in ETR) and in the NPQ may also have contributed to a higher production of ROS (Sergiev et al., 2006; Ahsan et al., 2008; Gomes and Juneau, 2016; Gomes et al., 2016a) due to over-excitation of chlorophylls (**Figure 6**, #7).

As we found in the present study (**Figure 3**; **Figure 6**, #8), increased lipid peroxidation has been previously observed in glyphosate-exposed plants and was related to increased H_2O_2 content in plants (Moldes et al., 2008; Miteva et al., 2010; Gomes et al., 2016b). Lipid peroxidation resulting from increased levels of ROS (such as H_2O_2) has been shown to affect the integrity of the thylakoid membranes (Richter, 1992), contributing to the noted decrease in ETR (**Figure 6**, #9). Glyphosate was demonstrated to cause depletion of photosynthetic proteins leading to losses of photosynthetic capacity in plants (Diaz Vivancos et al., 2011). However, it has long been recognized that H_2O_2 is a potent inhibitor of photosynthesis, since, even at low concentrations, it can inhibit CO_2 fixation by oxidizing the thiol groups of some essential enzymes of the Calvin cycle (Foyer and Noctor, 2011). We can therefore advance that the observed decrease in photosynthesis (ETR) in presence of glyphosate-based herbicide may also be directly linked to higher H_2O_2 concentration (**Figure 5**, #8). Carbon assimilation (and therefore photosynthesis) can also be negatively affected by the decreased

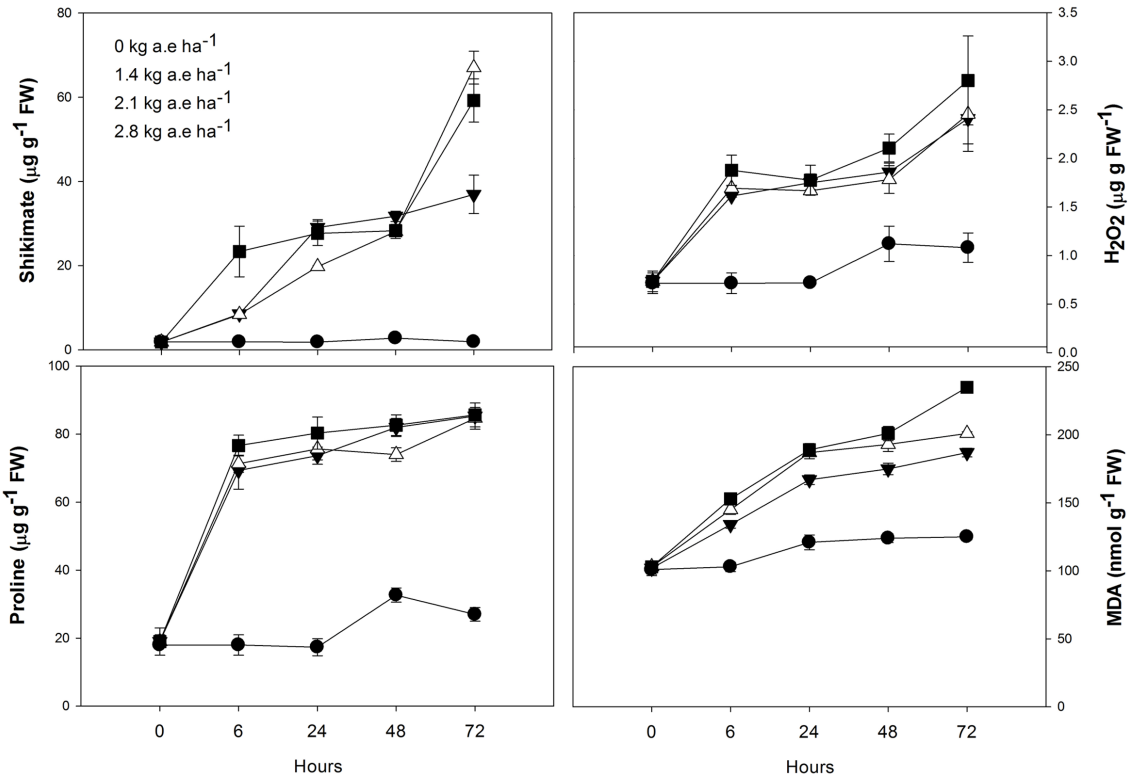


FIGURE 3 | Time courses of shikimate, proline and hydrogen peroxide concentrations, and lipid peroxidation (MDA concentrations) in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of increased (0, 1.4, 2.1, and 2.8 kg a.e ha^{-1}) rates of the glyphosate based herbicide (Factor® 540). Values are means ± SE of three replicates.

stomatal conductance (g_s) (**Figure 1**) in presence of herbicides (Zobiole et al., 2010). Reduced g_s, as also previously reported in *Hordeum vulgare* (barley) and *Lolium perenne* plants exposed to glyphosate (Olesen and Cedergreen, 2010; Yanniccari et al., 2012), can limit photochemistry, resulting in decreased ETR (**Figure 6**, #10). The observed ETR reduction could also be due to the alteration of the integrity of PSII (lower F_V/F_M) (**Figure 2**; **Figure 6**, #11). In addition, the decrease in total chlorophyll concentration in presence of the glyphosate-based herbicide may be responsible for a lower light interception and thus, the noted lower electron transport rate (**Figure 6**, #11). Decreased chlorophyll contents when plants are exposed to herbicide application have been demonstrated previously and have been attributed to an increase chlorophyll degradation or to a decrease in chlorophyll synthesis (Cakmak et al., 2009; Mateos-Naranjo et al., 2009; Huang et al., 2012; Gomes et al., 2016a).

In order to better understand the processes involved in H_2O_2 accumulation (and herbicide-induced oxidative damage), we investigated the activity of antioxidant system in treated plants. Increases in proline synthesis is a common protective-response of plants to stress conditions (Hayat et al., 2012). It is important to note, however, that proline can also act as a significant signaling molecule in plant physiological processes, mainly under stress conditions (Hare and Cress, 1997). In the present study, we suggest that the observed proline accumulation in treated plants is associated to oxidative protection, $NADP^+$ recovery and shikimate pathway stimulation (**Figure 6**, #12 and #13). As we also observed, proline biosynthesis is commonly stimulated by increased cellular-ROS concentration conditions (Soshinkova et al., 2013). Although proline can be synthesized from ornithine, metabolic labeling studies indicate that, under stress conditions, proline is mainly produced from glutamate (as reviewed by Hare and Cress, 1997). Therefore, the proline accumulation found in our study indicates that this pathway is highly activated (**Figure 6**, #12). A special function of proline in preventing oxidative damage and enhancing tolerance from abiotic oxidative stress has been proposed recently (Soshinkova et al., 2013) and proline accumulation in plants in response to glyphosate exposure was documented (Huang et al., 2012). Due to the loss of feedback control of the shikimate pathway by tyrosine (that regulates the activity of 3-deoxy-D-aravino-heptulosonate-7-phosphate synthase) (Crowley, 2006), the herbicide (glyphosate) led to an unregulated flux of carbon into the shikimate pathway (Siehl, 1997). As a result, there is an increased demand for erythrose-5-phosphate, the substrate of the first reaction of the shikimate pathway. Erythrose-5-phosphate is produced in the oxidative pentose phosphate pathway (OPPP), which is dependent on $NAD(P)^+$ availability and inhibited by NADPH (Hare and Cress, 1997). During proline synthesis, NADPH is

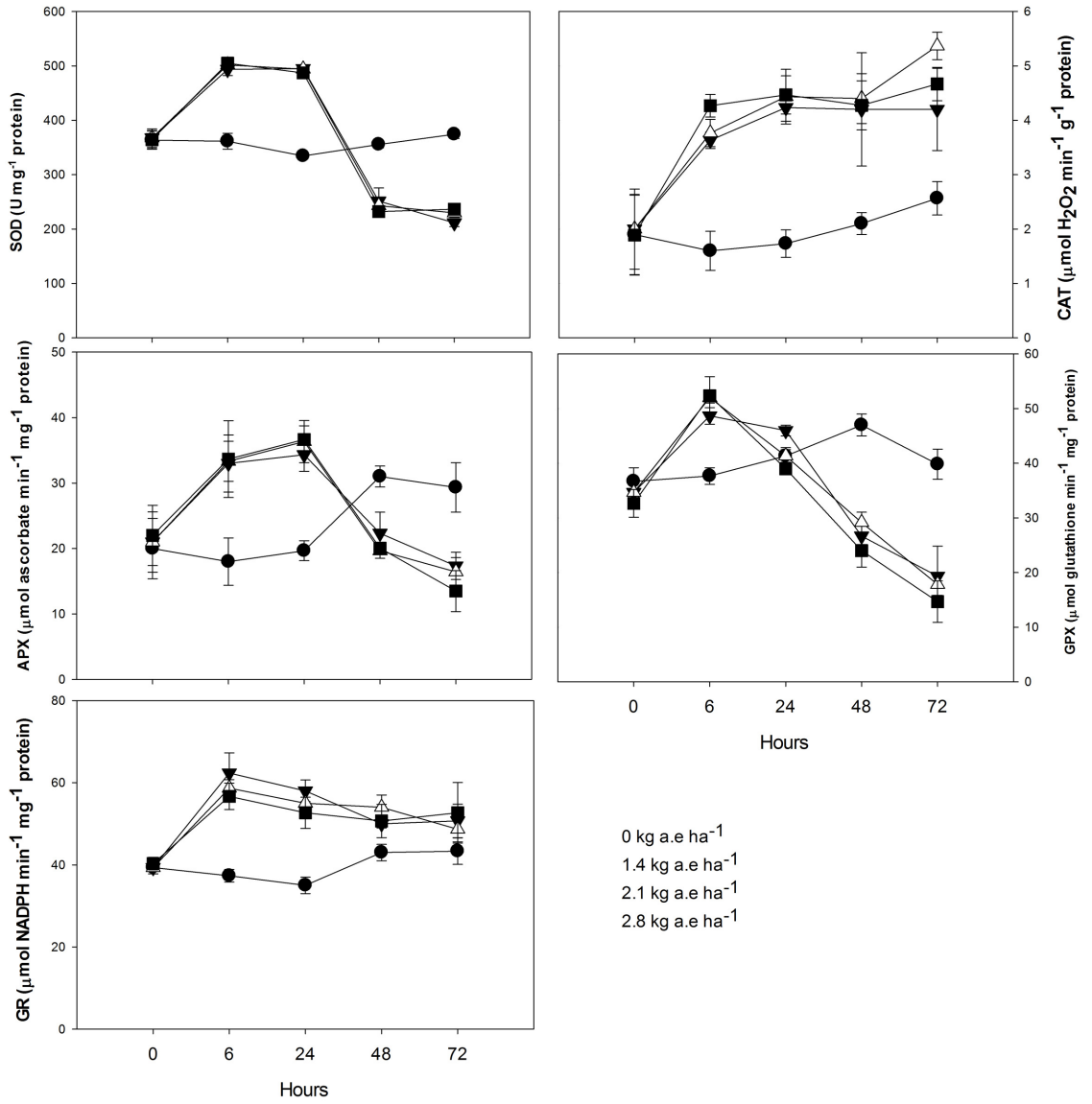


FIGURE 4 | Time courses of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR) activities in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of increased (0, 1.4, 2.1, and 2.8 kg a.e ha^{-1}) rates of the glyphosate based herbicide (Factor® 540). Values are means ± SE of three replicates.

oxidized, therefore stimulating OPPP. Even a small change in the NAD(P)$^+$/NADPH ratio may have a large effect on this redox-sensitive pathway (Hare and Cress, 1997). The oxidation of NADPH during proline synthesis, coupled with the reduction of NADP$^+$ during the two oxidative steps of the OPPP, promotes a cycle of changes in NAD(P)$^+$/NADPH ratio which stimulates proline biosynthesis, justifying its accumulation during stress (Hare and Cress, 1997). Therefore, upon the glyphosate-based herbicide exposure, the proline accumulation in willow plants could also be linked to the OPPP stimulation for the production of the erythrose-5-phosphate which will be used in shikimate pathway (**Figure 6**, #13). Supporting this hypothesis, Diaz Vivancos et al. (2011) observed decreased NADP/NADPH ratios in leaves of glyphosate-sensitive soybeans upon glyphosate treatment, as a result of the decreases in NADP$^+$ pool. As mentioned previously, under stress conditions, proline is mainly produced from glutamate (Hare and Cress, 1997). Glutamate is also required during δ-aminolevulinic acid (ALA; a chlorophyll precursor) biosynthesis through ALA-synthetase and γ,δ-dioxivalerate cycles (Beale, 1978). Therefore, if glutamate was preferentially used for proline biosynthesis (as suggested by proline greater accumulation in treated plants in relation to control; **Figure 3**), a decrease in ALA biosynthesis may be obtained, therefore contributing to the decreased chlorophyll concentration observed in treated plants (**Figure 6**, #14). As suggested by Gomes et al. (2016a), the decrease in chlorophyll

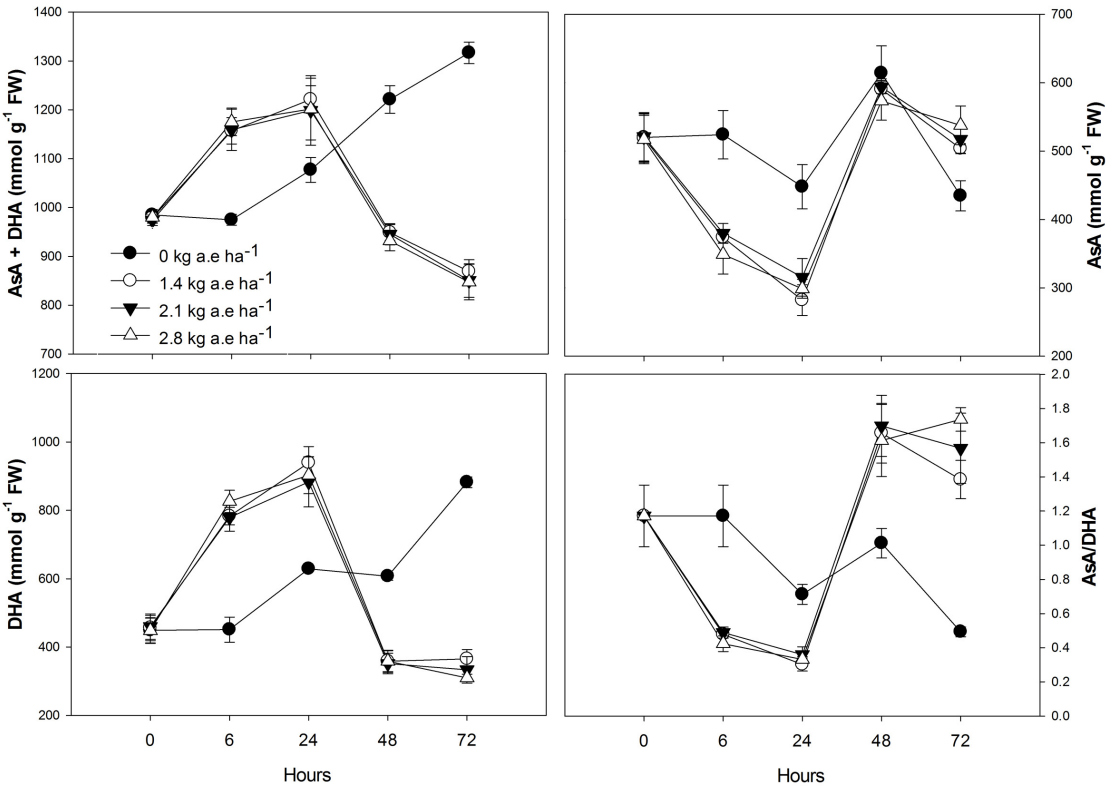


FIGURE 5 | Time courses of total ascorbate (AsA + DHA), reduced ascorbate (AsA), oxidized ascorbate (DHA), and AsA/DHA ratio in leaves of *Salix miyabeana* (cultivar SX64) plants spread with doses of increased (0, 1.4, 2.1, and 2.8 kg a.e ha^{-1}) rates of the glyphosate based herbicide (Factor® 540). Values are means ± SE of three replicates.

concentration may also be due to its degradation by increased ROS content (**Figure 6**, #15).

Even though treated plants showed increased activities of antioxidant enzymes after 6 h exposure, they were not able to prevent both peroxide accumulation and lipid peroxidation, indicating a clear deleterious effect of the glyphosate-based herbicide through oxidative burst. Moreover, the strong inhibition of SOD, APX, and GPX activities observed in plants exposed to herbicide after 48 h (**Figure 4**) can be related to the increased H_2O_2 and decreased ETR also observed in these plants. SOD is the first defense enzyme against oxidative stress (Pompeu et al., 2008) and is closely related to stress resistance in plants (Song et al., 2006). Indeed, this enzyme was involved in the PSII protection against the effects of prooxidant herbicides, limiting carbon dioxide and photoinhibitory conditions (Foyer et al., 1994; Arisi et al., 1998). The observed decrease in SOD activity (**Figure 4**) can therefore contribute to the herbicide-deleterious effects on photosynthesis in willow plants. We also demonstrated the key role of APX and GPX to prevent H_2O_2 accumulation in willow plants since: (1) decreased activities of both enzymes were related to increased H_2O_2 concentration in leaves; (2) even if treated plants shown higher CAT activity, it was not able to prevent H_2O_2 accumulation. The importance of APX and GR in avoiding oxidative stress has also been observed in metal(loid) treated plants (Chaoui et al., 1997; Gomes et al., 2013a,b) and the inactivation/degeneration of these enzymes has been related to increased H_2O_2 concentrations and oxidative damages to plants (Gomes et al., 2013b). When H_2O_2 accumulation exceeded the tolerance limit of plants, enzymatic systems are prone to protein carbonylation–an irreversible oxidative process in which the side chains of Lys, Arg, Pro, and Thr are converted to aldehyde or keto groups (Sohal et al., 2002), which may have been occurring in willow plants exposed to the studied herbicide (**Figure 6**, #16).

We also observed an interesting response of GR activity at 48 and 72 h, since its activity was not significantly decreased by the glyphosate-based herbicide exposure. GR is linked to APX and GPX activity by the glutathione-ascorbate cycle (Foyer and Noctor, 2011). However, as mentioned, the GR activity did not follow APX and GPX patterns upon the herbicide exposure. The maintenance of GR activity in treated plants indicates that APX and GPX activities were not limited by substrate availability, reinforcing that the proposed oxidative damage (protein carbonylation) of the enzymes could be responsible for their degeneration. We may hypothesize that, similarly to the proline production, the higher NADP(H)-dependent-GR activity can favor OPPP and contribute as a source of $NADP^+$ for photochemistry.

In addition to being the substrate for APX, ascorbate is an important antioxidant component of the cellular redox potential and its activity is linked to ascorbate-glutathione metabolic

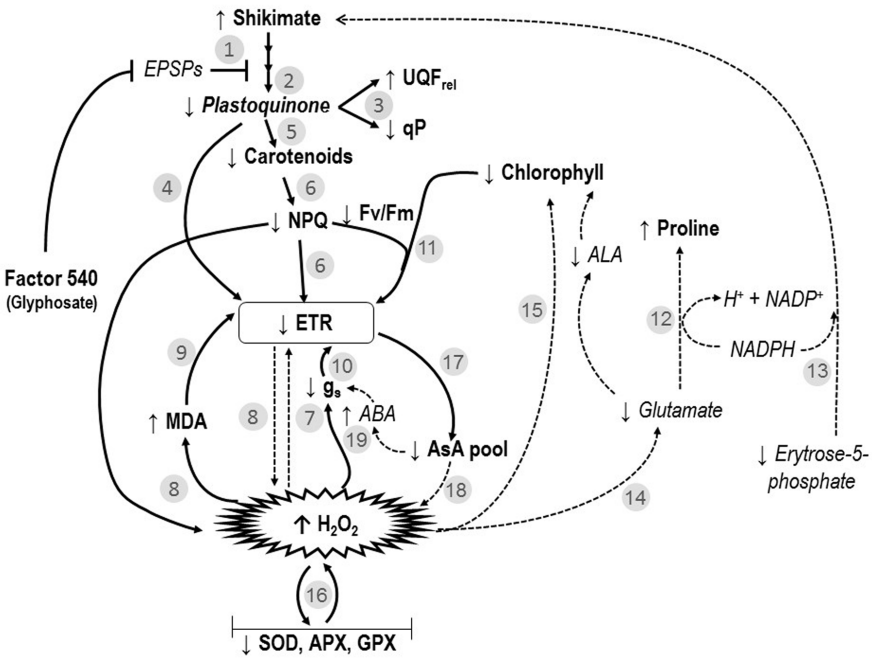


FIGURE 6 | Interconnected model of the effects of the glyphosate-based-herbicide (Factor® 540) on shikimate pathway, photosynthesis and oxidative markers of willow plants. Numbers refer to the ones mentioned in the discussion. ABA, abscisic acid; ALA, δ-aminolevulinic acid; APX, ascorbate peroxidase; AsA, ascorbate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; ETR, electron transport rate; F_V/F_M, maximal PSII photochemical efficiency; GPX, glutathione peroxidase; g_s, stomatal conductance; H_2O_2, peroxide; I_k, minimum saturating irradiance; MDA, lipid peroxidation; NPQ, non-photochemical quenching; qP, photochemical quenching; SOD, superoxide dismutase; UQF_{rel}, the relative unquenched fluorescence. Literature-based information in the models are expressed in italic words and in dotted arrows. While, observed data obtained in the present study are introduced in the model as bold words and non-dotted arrows.

cycle (Foyer and Noctor, 2011). In the present study, we found a link between the reduced form of ascorbate (AsA) and the APX activity. Indeed, up to 24 h, treated plants showed higher APX activity concomitantly to the reduced AsA concentration in their leaves; similarly, decreased APX activity for the following treatment periods was related to the increased AsA content. On the other hand, the contrary was observed for the oxidized form of ascorbate (DHA). The accumulation of AsA, as noted by the increased AsA/DHA ratio in treated plants, shows that the DHA has been effectively recycled to AsA by ascorbate-glutathione cycle. We also observed that total ascorbate concentrations (AsA + DHA) were reduced in herbicide treated plants (**Figure 5**). It is known that ascorbate concentration and ETR are closely linked, as the light-dependent stimulation of ascorbate biosynthesis requires photosynthetic electron transport activity (Yabuta et al., 2007). Thus, reduced ETR in treated plants could explain the observed reduction in ascorbate pool (**Figure 6**, #17). Low ascorbate pool favors the increase in both ROS (**Figure 6**, #18) and abscisic acid (ABA), leading to an increase in signal transduction through ROS-mediated and ABA-dependent signaling cascades (Foyer and Noctor, 2011). Among others, the interactive effect of ROS and ABA in stomatal movement is well studied, with increased ROS and ABA content inducing stomatal closure (Gomes et al., 2014a). This mechanism can also be related to the observed herbicide-induced decreases in g_s (**Figure 6**, #19).

As expected, the primary target site of the studied glyphosate-based-herbicide (Factor® 540) on willow plants is the shikimate pathway. We demonstrated, for the first time, that on top of the alteration of this primary target site, this herbicide induces a series of interconnected events that leads to decreased photosynthetic activity in willow plants. Furthermore, we showed that the herbicide-deleterious effects on photosynthesis are strongly related to herbicide-induced oxidative stress, and that reduction of photosynthesis may amplify the observed effect by inducing ROS production. Our results evidenced that as for photosynthesis-target herbicides, which trigger ROS production and oxidative stress, glyphosate herbicidal effect may be related to induction of ROS accumulation. The inhibition of shikimate pathway may induce changes in redox status with important consequences in leaf metabolism, mainly on photosynthesis. Glyphosate tolerance in plants, for instance, have been related to the ability of plants to deal with ROS accumulation through the activation of antioxidant systems (Maroli et al., 2015). However, since photosynthetic processes of GR plants have been shown to be affected by glyphosate-based herbicides (Zobiole et al., 2010, 2011, 2012), glyphosate may target other cellular sites, inducing ROS formation, for example, mitochondrial electron chain, as proposed by Gomes and Juneau (2016). Although ROS formation may also be produced in the mitochondria, our model fits with several results presented in the literature about the effects

of glyphosate in sensitive plants, highlighting the role of ROS induction in this herbicidal mechanism of action.

AUTHOR CONTRIBUTIONS

MG, SL performed the experiments; MG, MLa, and PJ designed the experiments; MG and PJ wrote the paper; LH-E and MLu gave technical support and conceptual advice.

FUNDING

This research was supported by the Natural Science and Engineering Research Council of Canada (NSERC) through a Strategic grant awarded to MLa, PJ, and MLu. MG received a Ph.D. scholarship from Fonds de Recherche du Québec–Nature et Technologies (FRQNT) and LH-E received a Ph.D. scholarship from the Natural Science and Engineering Research Council of Canada (NSERC).

ACKNOWLEDGMENT

We thank Jerzy Kruk from the Department of Plant Physiology and Biochemistry, Jagiellonian University for providing us the plastoquinone standard.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00207/full#supplementary-material

REFERENCES

Ahsan, N., Lee, D.-G., Lee, K.-W., Alam, I., Lee, S.-H., Bahk, J. D., et al. (2008). Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach. *Plant Physiol. Biochem.* 46, 1062–1070. doi: 10.1016/j.plaphy.2008.07.002

Aniya, Y., and Anders, M. W. (1992). Activation of rat liver microsomal glutathione S-transferase by hydrogen peroxide: role for protein-dimer formation. *Arch. Biochem. Biophys.* 296, 611–616. doi: 10.1016/0003-9861(92)90617-6

Arisi, A., Cornic, G., Jouanin, L., and Foyer, C. (1998). Overexpression of iron superoxide dismutase in transformed poplar modifies the regulation of photosynthesis at low CO2 partional pressures or following exposure to prooxidant methyl viologen. *Plant Physiol.* 117, 565–574. doi: 10.1104/pp.117.2.565

Bates, L. S., Waldren, R. P., and Teare, I. (1973). Rapid determination of free proline for water stress studies. *Plant Soil* 39, 205–208. doi: 10.1016/j.dental.2010.07.006

Beale, S. (1978). S-aminolevulinic acid in plants: its biosynthesis, regulation, and role in plastid development. *Annu. Rev. Plant Physiol.* 29, 95–120. doi: 10.1146/annurev.pp.29.060178.000523

Bijay, K. S., and Dale, L. S. (1998). Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. *Weed Sci. Soc. Am.* 12, 527–530.

Bott, S., Tesfamariam, T., Kania, A., Eman, B., Aslan, N., Römheld, V., et al. (2011). Phytotoxicity of glyphosate soil residues re-mobilised by phosphate fertilisation. *Plant Soil* 342, 249–263. doi: 10.1007/s11104-010-0689-3

Boussiba, S. (2000). Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiol. Plant.* 108, 111–117. doi: 10.1034/j.1399-3054.2000.108002111.x

Bouvier, F., Backhaus, R. A., Camara, B., and Chem, J. B. (1998). Induction and control of chromoplast-specific carotenoid genes by oxidative stress induction and control of chromoplast-specific carotenoid genes by oxidative stress. *J. Biol. Chem.* 273, 30651–30659. doi: 10.1074/jbc.273.46.30651

Bouvier, F., D'Harlingues, A., Hugueney, P., Marin, E., Marion-Poll, A., and Camara, B. (1996). Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta -cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *J. Biol. Chem.* 271, 28861–28867. doi: 10.1074/jbc.271.46.28861

Cakmak, I., Yazici, A., Tutus, Y., and Ozturk, L. (2009). Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. *Eur. J. Agron.* 31, 114–119. doi: 10.1016/j.eja.2009.07.001

Cerdeira, A. L., and Duke, S. O. (2006). The current status and environmental impacts of glyphosate-resistant crops: a review. *J. Environ. Qual.* 35, 1633–1658. doi: 10.2134/jeq2005.0378

Chaoui, A., Habib Ghorbal, M., and El Ferjani, E. (1997). Effects of cadmium-zinc interactions on hydroponically grown bean (*Phaseolus vulgaris* L.). *Plant Sci.* 126, 21–28. doi: 10.1016/S0168-9452(97)00090-3

Coupe, R., Kalkhoff, S., Capel, P., and Gregoire, C. (2012). Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe. *Geophys. Res. Abstr.* 14, 5877.

Crowley, V. (ed.). (2006). *The Isozymes of 3-Deoxy-D-Arabino-Heptulosonate 7-Phosphate Synthase from Arabidopsis Perform Differential and Overlapping Roles in Vivo and May be Regulated by Tyrosine.* Toronto: University of Toronto.

Demming-Adams, B., and Adams, W. (2000). Photosynthesis: harvesting sunlight safety. *Nature* 403, 371–374. doi: 10.1038/35000315

Diaz Vivancos, P., Driscoll, S. P., Bulman, C. A., Ying, L., Emami, K., Treumann, A., et al. (2011). Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. *Plant Physiol.* 157, 256–268. doi: 10.1104/pp.111.181024

Duke, S. O., and Powles, S. B. (2008). Glyphosate: a once-in-a-century herbicide. *Pest Manag. Sci.* 64, 319–325. doi: 10.1002/ps.1518

Eilers, P. H. C., and Peeters, J. C. H. (1988). A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. *Ecol. Modell.* 42, 199–215. doi: 10.1016/0304-3800(88)90057-9

Fan, L., Vonshak, A., Zarka, A., and Boussiba, S. (1998). Does astaxanthin protect *Haematococcus* against light damage? *Z. Naturforsch. C.* 53, 93–100.

Fedtke, K., and Duke, S. (2005). "Herbicides," in *Plant Toxicology*, eds B. Hock and E. Elstner (New York, NY: Marcel Dekker), 247–330.

Foyer, C. H., Leiandais, M., and Kunert, K. J. (1994). Photooxidative stress in plants. *Physiol. Plant.* 92, 696–717. doi: 10.1111/j.1399-3054.1994.tb03042.x

Foyer, C. H., and Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 155, 2–18. doi: 10.1104/pp.110.167569

Gomes, M. P., Carvalho, M., Carvalho, G. S., Garcia, Q. S., Guilherme, L. R. G., and Soares, A. M. (2013a). Phosphorus improves arsenic phytoremediation by *Anadenanthera peregrina* by alleviating induced oxidative stress. *Int. J. Phytoremediation* 15, 633–646.

Gomes, M. P., Duarte, D. M., Carneiro, M. M. L. C., Barreto, L. C., Carvalho, M., Soares, A. M., et al. (2013b). Zinc tolerance modulation in *Myracrodruon urundeuva* plants. *Plant Physiol. Biochem.* 67, 1–6. doi: 10.1016/j.plaphy.2013.02.018

Gomes, M. P., and Juneau, P. (2016). Oxidative stress in duckweed (*Lemna minor* L.) induced by glyphosate: Is the mitochondrial electron transport chain a target of this herbicide?. *Environ. Pollut.* 218, 402–409. doi: 10.1016/j.envpol.2016.07.019

Gomes, M. P., Le Manac'h, S. G., Maccario, S., Labrecque, M., Lucotte, M., and Juneau, P. (2016a). Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll

metabolism in willow plants. *Pestic. Biochem. Physiol.* 130, 65–70. doi: 10.1016/j.pestbp.2015.11.010

Gomes, M. P., Le Manac'h, S. G., Moingt, M., Smedbol, E., Paquet, S., Labrecque, M., et al. (2016b). Impact of phosphate on glyphosate uptake and toxicity in willow. *J. Hazard. Mater.* 304, 269–279. doi: 10.1016/j.jhazmat.2015.10.043

Gomes, M. P., Smedbol, E., Carneiro, M. M. L. C., Garcia, Q. S., and Juneau, P. (2014a). "Reactive oxygen species and plant hormones," in *Oxidative Damage to Plants: Antioxidant Networks and Signaling*, ed. P. Ahmad (New York, NY: Academic Press), 65–81.

Gomes, M. P., Smedbol, E., Chalifour, A., Hénault-Ethier, L., Labrecque, M., Lepage, L., et al. (2014b). Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid (AMPA), an overview. *J. Exp. Bot.* 65, 4691–4703. doi: 10.1093/jxb/eru269

Gomes, M. P., Soares, A. M., and Garcia, Q. S. (2014c). Phosphorous and sulfur nutrition modulate antioxidant defenses in *Myracrodruom urundeuva* plants exposed to arsenic. *J. Hazard. Mater.* 276, 97–104. doi: 10.1016/j.jhazmat.2014.05.020

Gunes, A., Inal, A., Bagci, E. G., Coban, S., and Pilbeam, D. J. (2007). Silicon mediates changes to some physiological and enzymatic parameters symptomatic for oxidative stress in spinach (*Spinacia oleracea* L.) grown under B toxicity. *Sci. Hortic.* 113, 113–119. doi: 10.1016/j.scienta.2007.03.009

Hare, P. D., and Cress, W. A. (1997). Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.* 21, 79–102. doi: 10.1016/j.plaphy.2013.05.028

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal. Behav.* 7, 1456–1466. doi: 10.4161/psb.21949

Huang, J., Silva, E. N., Shen, Z., Jiang, B., and Lu, H. (2012). Effects of glyphosate on photosynthesis, chlorophyll fluorescence and physicochemical properties of cogongrass (*Imperata cylindrical* L.). *Plant Omi. J.* 5, 177–183.

Inderjit, and Kaushik, S. (2010). Effect of herbicides with different modes of action on physiological and cellular traits of Anabaena fertilissima. *Paddy Water Environ.* 8, 277–282. doi: 10.1007/s10333-010-0208-4

Juneau, P., Barnett, A., Méléder, V., Dupuy, C., and Lavaud, J. (2015). Combined effect of high light and high salinity on the regulation of photosynthesis in three diatom species belonging to the main growth forms of intertidal flat inhabiting microphytobenthos. *J. Exp. Mar. Biol. Ecol.* 463, 95–104. doi: 10.1016/j.jembe.2014.11.003

Juneau, P., Green, B. R., and Harrison, P. J. (2005). Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: limitations of some PAM-parameters in studying environmental stress effects. *Photosynthetica* 43, 75–83. doi: 10.1007/s11099-005-5083-7

Kitajima, M., and Butler, W. L. W. (1975). Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochem. Biophys. Acta* 376, 105–115. doi: 10.1016/0005-2728(75)90209-1

Krall, J. P., and Edwards, G. E. (1992). Relationship between photosystem II activity and CO2 fixation in leaves. *Physiol. Plant.* 86, 180–187. doi: 10.1111/j.1399-3054.1992.tb01328.x

Kruk, J., and Karpinski, S. (2006). An HPLC-based method of estimation of the total redox state of plastoquinone in chloroplasts, the size of the photochemically active plastoquinone-pool and its redox state in thylakoids of *Arabidopsis*. *Biochim. Biophys. Acta* 1757, 1669–1675. doi: 10.1016/j.bbabio.2006.08.004

Labrecque, M., and Teodorescu, T. I. (2005). Field performance and biomass production of 12 willow and poplar clones in short-rotation coppice in southern Quebec (Canada). *Biomass Bioenergy* 29, 1–9. doi: 10.1016/j.biombioe.2004.12.004

Lee, T. T. (1981). Effects of glyphosate on synthesis and degradation of chlorophyll in soybean and tobacco cells. *Weed Res.* 21, 161–164. doi: 10.1111/j.1365-3180.1981.tb00111.x

Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592. doi: 10.1042/bst0110591

Maroli, A. S., Nandula, V. K., Dayan, F. E., Duke, S. O., Gerard, P., and Tharayil, N. (2015). Metabolic profiling and enzyme analyses indicate a potential role of antioxidant systems in complementing glyphosate resistance in an *Amaranthus palmeri* Biotype. *J. Agric. Food Chem.* 63, 9199–9209. doi: 10.1021/acs.jafc.5b04223

Mateos-Naranjo, E., Redondo-Gómez, S., Cox, L., Cornejo, J., and Figueroa, M. E. (2009). Effectiveness of glyphosate and imazamox on the control of the invasive cordgrass *Spartina densiflora*. *Ecotoxicol. Environ. Saf.* 72, 1694–1700. doi: 10.1016/j.ecoenv.2009.06.003

Maxwell, K., and Johnson, G. N. (2000). Chlorophyll fluorescence–a practical guide. *J. Exp. Bot.* 51, 659–668. doi: 10.1093/jexbot/51.345.659

Miteva, L.-E. L. P.-E., Ivanov, S. V., and Alexieva, V. S. (2010). Alterations in glutathione pool and some related enzymes in leaves and roots of pea plants treated with the herbicide glyphosate. *Russ. J. Plant Physiol.* 57, 131–136. doi: 10.1134/S1021443710010188

Moldes, C. A., Medici, L. O., Abrahão, O. S., Tsai, S. M., and Azevedo, R. A. (2008). Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. *Acta Physiol. Plant.* 30, 469–479. doi: 10.1007/s11738-008-0144-8

Olesen, C. F., and Cedergreen, N. (2010). Glyphosate uncouples gas exchange and chlorophyll fluorescence. *Pest Manag. Sci.* 66, 536–542. doi: 10.1002/ps.1904

Pompeu, G. B., Gratão, P. L., Vitorello, V. A., and Azevedo, R. A. (2008). Antioxidant isoenzyme responses to nickel-induced stress in tobacco cell suspension culture. *Sci. Agric.* 65, 548–552. doi: 10.1590/S0103-90162008000500015

Redondo-Gómez, S., Mateos-Naranjo, E., Cambrollé, J., Luque, T., Figueroa, M. E., and Davy, A. J. (2008). Carry-over of differential salt tolerance in plants grown from dimorphic seeds of *Suaeda splendens*. *Ann. Bot.* 102, 103–112. doi: 10.1093/aob/mcn069

Richter, C. (1992). Reactive oxygen and DNA damage in mitochondria. *Mutat. Res.* 275, 249–255. doi: 10.1016/0921-8734(92)90029-O

Sandmann, G., Römer, S., and Fraser, P. D. (2006). Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab. Eng.* 8, 291–302. doi: 10.1016/j.ymben.2006.01.005

Sergiev, I. G., Alexieva, V. S., Ivanov, S., Moskova, I. I., and Karanov, E. N. (2006). The phenylurea cytokinin 4PU-30 protects maize plants against glyphosate action. *Pestic. Biochem. Physiol.* 85, 139–146. doi: 10.1016/j.pestbp.2006.01.001

Serra, A.-A., Nuttens, A., Larvor, V., Renault, D., Couée, I., Sulmon, C., et al. (2013). Low environmentally relevant levels of bioactive xenobiotics and associated degradation products cause cryptic perturbations of metabolism and molecular stress responses in *Arabidopsis thaliana*. *J. Exp. Bot.* 64, 2753–2766. doi: 10.1093/jxb/ert119

Servaites, J. C., Tucci, M. A., and Geiger, D. R. (1987). Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves. *Plant Physiol.* 85, 370–374. doi: 10.1104/pp.85.2.370

Siehl, D. (1997). "Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis," in *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*, eds R. Roe, J. Burton, and R. Kuhr (Amsterdam: IOS Press), 37–67.

Sohal, R. S., Mockett, R. J., and Orr, W. C. (2002). Mechanisms of aging: an appraisal of the oxidative stress hypothesis. *Free Radic. Biol. Med.* 33, 575–586. doi: 10.1016/S0891-5849(02)00886-9

Song, F. N., Yang, C. P., Liu, X. M., and Liu, G. B. (2006). Effect of salt stress on activity of superoxide dismutase (SOD) in Ulmus primula L. *J. For. Res.* 17, 13–16. doi: 10.1007/s11676-006-0003-7

Soshinkova, T. N., Radyukina, N. L., Korolkova, D. V., and Nosov, A. V. (2013). Proline and functioning of the antioxidant system in *Thellungiella* salsuginea plants and cultured cells subjected to oxidative stress. *Russ. J. Plant Physiol.* 60, 41–54. doi: 10.1134/S1021443713010093

Su, Y. S., Ozturk, L., Cakmak, I., and Budak, H. (2009). Turfgrass species response exposed to increasing rates of glyphosate application. *Eur. J. Agron.* 31, 120–125. doi: 10.1016/j.eja.2009.05.011

van Kooten, O., and Snel, J. (1990). The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res.* 25, 147–150. doi: 10.1007/BF00033156

Vendrell, E., Gómez de Barreda Ferraz, D., Sabater, C., and Carrasco, J. M. (2009). Glyphosate on growth of four freshwater species of phytoplankton: a microplate bioassay. *Bull. Environ. Contam. Toxicol.* 82, 538–542. doi: 10.1007/s00128-009-9674-z

Walter, A., Rascher, U., and Osmond, B. (2003). Transitions in photosynthetic parameters of midvein and interveinal regions of leaves and their importance during leaf growth and development. *Plant Biol. (Stuttg)* 6, 184–191. doi: 10.1055/s-2004-817828

Wang, C.-Y. (2001). Effect of glyphosate on aromatic amino acid metabolism in purple Nutsedge (*Cyperus rotundus*)1. *Weed Technol.* 15, 628–635. doi: 10.1614/0890-037X(2001)015[0628:EOGOAA]2.0.CO;2

Williams, G. M., Kroes, R., and Munro, I. C. (2000). Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regul. Toxicol. Pharmacol.* 31, 117–165. doi: 10.1006/rtph.1999.1371

Yabuta, Y., Mieda, T., Rapolu, M., Nakamura, A., Motoki, T., Maruta, T., et al. (2007). Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in *Arabidopsis*. *J. Exp. Bot.* 58, 2661–2671. doi: 10.1093/jxb/erm124

Yanniccari, M., Tambussi, E., Istilart, C., and Castro, A. M. (2012). Glyphosate effects on gas exchange and chlorophyll fluorescence responses of two *Lolium perenne* L. biotypes with differential herbicide sensitivity. *Plant Physiol. Biochem.* 57, 210–217. doi: 10.1016/j.plaphy.2012.05.027

Yoshida, K., Shibata, M., Terashima, I., and Noguchi, K. (2010). Simultaneous determination of in vivo plastoquinone and ubiquinone redox states by HPLC-based analysis. *Plant Cell Physiol.* 51, 836–841. doi: 10.1093/pcp/pcq044

Zobiole, L. H. S., de Oliveira, R. S., Kremer, R. J., Constantin, J., Bonato, C. M., and Muniz, A. S. (2010). Water use efficiency and photosynthesis of glyphosate-resistant soybean as affected by glyphosate. *Pestic. Biochem. Physiol.* 97, 182–193. doi: 10.1016/j.pestbp.2010.01.004

Zobiole, L. H. S., Kremer, R. J., de Oliveira, R. S. Jr., and Constantin, J. (2012). Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. *J. Plant Nutr. Soil Sci.* 175, 319–330. doi: 10.1002/jpln.201000434

Zobiole, L. H. S. S., Kremer, R. J., Oliveira, R. S. Jr., Constantin, J., and Oliveira, R. S. (2011). Glyphosate affects chlorophyll, nodulation and nutrient accumulation of "second generation" glyphosate-resistant soybean (*Glycine max* L.). *Pestic. Biochem. Physiol.* 99, 53–60. doi: 10.1016/j.pestbp.2010.10.005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Gomes, Le Manac'h, Hénault-Ethier, Labrecque, Lucotte and Juneau. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 28 March 2017
doi: 10.3389/fenvs.2017.00010

Effects of the Herbicide Metsulfuron-Methyl on a Plant Community, Including Seed Germination Success in the F1 Generation

J. Bas Nelemans[1,2], *René P. A. van Wijngaarden*[2], *Ivo Roessink*[2] *and Gertie H. P. Arts*[2]*

[1] *Plant Ecology and Nature Conservation, Wageningen University, Wageningen, Netherlands,* [2] *Environmental Risk Assessment, Wageningen Environmental Research (Alterra), Wageningen University and Research, Wageningen, Netherlands*

A field trial was set up to simulate a field margin environment to analyze sub-lethal effects of the herbicide metsulfuron-methyl on several endpoints of non-target terrestrial plants (NTTPs). Both vegetative and reproductive endpoints were evaluated. The experiment was conducted in an experimentally established field strip with sown species. The treatments consisted of five dosages and a control: 0, 0.0097, 0.0193, 0.058, 0.174, and 0.348 gram active ingredient per hectare (g a.i./ha). The plant cover, number of (flowering) individuals per species and fruit collection were performed and estimated weekly for a period of 4 months. At the end of the growing season, the total dry biomass per species was obtained and the collected fruits were weighed, counted, and sieved to obtain the seeds. The seeds were counted and weighed as well, before they were used in a germination experiment to test the seed emergence of the F1 generation. The herbicide only affected the biomass of *Matricaria recutita* at the treatment levels tested (0.058 g a.i./ha and higher). Field dosages of 0.174 and 0.348 g a.i./ha differed significantly in the endpoint "plant cover" compared to lower dosages and controls. The F1 generations of *Sinapis alba*, *Centaurea cyanus,* and *Phacelia tanacetifolia* were particularly affected at field dosages of 0.0193 g a.i./ha and higher, showing significantly lower seed germination rates. This would imply that spray drift of metsulfuron-methyl might lead to shifts in species compositions and succession in vegetation in off-crop areas adjacent to arable fields. Conducting germination experiments is necessary to investigate a herbicide's effect on the full life cycle of plants.

Keywords: non-target terrestrial plants, metsulfuron-methyl, field trial, reproductive endpoints, seed germination, ecological risk assessment

OPEN ACCESS

Edited by:
Carsten A. Brühl, University of Koblenz and Landau, Germany

Reviewed by:
Céline Boutin, Government of Canada, Canada
Ilias Travlos, Agricultural University of Athens, Greece

***Correspondence:**
Gertie H. P. Arts
gertie.arts@wur.nl

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *29 November 2016*
Accepted: *03 March 2017*
Published: *28 March 2017*

Citation:
Nelemans JB, van Wijngaarden RPA, Roessink I and Arts GHP (2017) Effects of the Herbicide Metsulfuron-Methyl on a Plant Community, Including Seed Germination Success in the F1 Generation. Front. Environ. Sci. 5:10. doi: 10.3389/fenvs.2017.00010

INTRODUCTION

In recent decades, a decline in the diversity and abundance of wild plants in agro-systems has been reported in both Europe and North America (Geiger et al., 2010; Strandberg et al., 2012; Boutin et al., 2014). This is a worrying trend, as these agro-systems harbor a significant proportion of the overall plant biodiversity (Robinson and Sutherland, 2002). One of the causes of this

decline is the increased use of herbicides (Geiger et al., 2010). Applying herbicides is a common method to maximize the yield of crops by suppressing the growth of unwanted wild species competing for the same resources (e.g., space, light, nutrients). However, fractions of the herbicide may end up in adjacent terrestrial sites, where weed control is not intended (Boutin and Rogers, 2000), e.g., due to spray drift. The plants occurring at these sites, called non-target terrestrial plants (NTTPs), need to be protected in view of their important functions in ecosystems, as described in the plant protection goals published by the European Food Safety Authority (EFSA PPR Panel, 2014). To assess the risks of herbicides for NTTPs, two guidelines are available, focusing on tests in laboratory and greenhouse environments (OECD 208, 2006; OECD 227, 2006). However, there is almost no guidance or information available on how to study herbicide impact on NTTPs in the field (OECD 208, 2006; OECD 227, 2006; EFSA PPR Panel, 2014; Schmitz et al., 2015). Spray drift is considered to be the most important source of pollution for NTTPs adjacent to crop fields (EFSA PPR Panel, 2014; Arts et al., 2015).

Sulfonylureas represent one of the largest classes of herbicides, with at least 27 different active ingredients (a.i.) registered around the world (Russell et al., 2002). One of the sulfonylurea compounds is metsulfuron-methyl ($C_{14}H_{15}N_5O_6S$), known to control 60 species as a potent inhibitor of plant growth and is especially used for the control of dicotyledon species (Boutin et al., 2004, 2012). Its mode of action involves limiting cell division (Russell et al., 2002).

The risks of spray drift of herbicides to NTTPs is assessed using plant characteristics (or endpoints), sensitive to the mode of action. Endpoints are plant characteristics that are sensitive to the mode of action of the chemical being used (EFSA PPR Panel, 2014). Risk estimates have been based on ED50 (the dosage at which the specific endpoint value decreases by 50%) for the various endpoints selected. In the official guidelines of the Organization for Economic Co-operation and Development (OECD), biomass is used as a vegetative endpoint to assess the risks (OECD 208, 2006; OECD 227, 2006). However, reproductive endpoints such as seed and fruit yield are also important to include in these risk assessments. For example, in the field study by Kjær et al. (2006), exposure of Hawthorn *(Crateaegus monogyna)* to metsulfuron-methyl resulted in 100% losses of berries at a field application rate of 0.05 g a.i./ha, while the vegetative endpoints (leaves) remained unaffected. Findings of other studies (Boutin et al., 2000; Blackburn and Boutin, 2003; Olszyk et al., 2004; Carpenter and Boutin, 2010; Pfleeger et al., 2012; EFSA PPR Panel, 2014; Schmitz et al., 2014, 2015) also suggest that reproductive endpoints are more sensitive than vegetative endpoints, especially in comparison with biomass as a vegetative endpoint.

Studies of NTTPs and herbicides which only include vegetative endpoints to assess the risk are thus not sufficient to test the overall effect of a herbicide on the whole life cycle of a plant, as the reproductive endpoints may be a crucial factor for the persistence of natural plant populations. Important stages of a natural plant population are the germination of seeds, seedling and juvenile stages, flowering, seed production and the germination rates of these seeds (EFSA PPR Panel, 2014). Completion of the full life cycle of a plant is necessary for it to contribute to a sustainable population, the latter being defined as one of the protection goals by EFSA. The aim of the present study was to investigate whether the sub-lethal dosages of metsulfuron methyl that are currently applied cause effects on plant populations in the field. Several studies (Blackburn and Boutin, 2003; Rokich et al., 2009; EFSA PPR Panel, 2014) investigated the germination of seeds from plants exposed in their mature stage, while none investigated the germination of seeds from plants exposed in their juvenile stage. Studies with juvenile stages could be important as the exposure to most herbicides in real agro-systems might occur when the plants are in their juvenile stage (Arts et al., 2015), however this is not always the case (Strandberg et al., 2012; Boutin et al., 2014).

The present field trial assessed the effects of the herbicide metsulfuron-methyl on seven terrestrial plant species in a field margin freshly sown with a mixture of wild plants. We tried to answer the following research questions: (1) How does the sensitivity of reproductive endpoints differ from that of vegetative endpoints? (2) Is there an effect on the next generation of herbicide-exposed NTTPs? (3) How do species differ in their sensitivity to the herbicide sprayed at environmentally realistic exposure rates?

MATERIALS AND METHODS

Location

The field trial was carried out at the Sinderhoeve research station in Renkum, The Netherlands. The soil was mainly sandy, well-drained and moderately rich in nutrients. The field margin had a length of 100 m and was 8 m wide. The field margin was larger than the actual plots used for monitoring and sampling (see Supporting Information) in order to prevent intrusion of plants from the surrounding area. The immediately surrounding environment area consisted of a regularly mown unsprayed grassland.

Seed Mixture

A seed mixture containing 11 species was used to establish the vegetation in the 8 × 100 m field margin. The field margin consisted of bare soil at the time of sowing. The mixture of seeds (200 g per species) was evenly spread over the field margin by means of a sower and subsequently harrowed. The commercially available mixture (**Table 1**) consisted of both annual and perennial species, to simulate a realistic situation. The mixture contained several families which are highly susceptible to the herbicide metsulfuron-methyl like *Brassicaceae* and *Fabaceae* (Boutin et al., 2000, 2004). Of the 11 sown species, only six species developed to a mature stage (**Table 1**). Furthermore, *Lupinus perennis* and *Melilotus officinalis* did not produce seeds within the experimental period. In contrast, *Glebionis segetum*, which was not part of the seed mixture, developed rapidly during the growing season from the existing seed bank. This species was therefore also included as a seventh species in the analysis. The seed mixture was sown on June 17, 2015. The experiment lasted from July 16 to the first week of October 2015.

TABLE 1 | List of plant species sown (x, present; –, not present; P, perennial; A, annual; B, biennial).

Common name	Scientific name	Family	Presence in field	Life cycle
Chamomile	*Matricaria recutita* L.	*Asteraceae*	x	A
Cornflower	*Centaurea cyanus* L.	*Asteraceae*	x	A
Yellow mustard	*Sinapis alba* L.	*Brassicaceae*	x	A
Phacelia	*Phacelia tanacetifolia* Benth.	*Boraginaceae*	x	A
Peach-leaved bellflower	*Campanula persicifolia* L.	*Campanulaceae*	–	P
Corn poppy	*Papaver rhoeas* L.	*Papaveracea*	–	A
Yellow sweet clover	*Melilotus officinalis* L. (Pall.)	*Fabaceae*	x	A or B
Forking Larkspur	*Consolida regalis* Gray	*Ranunculaceae*	–	A
Parsnip	*Pastinaca sativa* L.	*Apiaceae*	–	P
Wild perennial lupine	*Lupinus perennis* L.	*Fabaceae*	x	B
Buttercup	*Ranunculus acris* L.	*Ranunculaceae*	–	P

Experimental Set-Up and Measurements

The set-up used a replicated block design (four blocks with increasing dosages; see **Figure 1**). Vegetative and reproductive endpoints were assessed in separate parts of the plots. The northern half of each plot in the field margin was used to determine the total aboveground biomass for each species (i.e., the species sown and developed from the seed mixture and *G. segetum*). The plants were harvested in the first week of October 2015, in two 0.5 m^2 sub-plots per plot. The fresh plant material was first sorted by species, after which the fresh biomass was determined in the lab. Subsequently, the plant material was dried in an oven at 70°C for 2 days, and the dry biomass was determined for each species.

The southern half of the margin was used to collect seeds and fruits as reproductive endpoints. In addition, this part of the field margin was also used to estimate the plant cover and to count the number of flowering and non-flowering individuals per species. These measurements were performed weekly from July till first week of October 2015 using an 0.25 m^2 plot. Each measurement was based on three sub-plots, which corresponded to three measurements in each plot, using a quadrat. If an individual plant was producing seeds, its fruits were harvested and stored dry in marked paper bags at room temperature. After all fruits had been harvested, the seeds were counted and weighed after physically extracting them from the fruits using sieves and an air extractor. The sieves had a mesh size ranging from 0.5 to 2 mm. After this extraction, all seeds of *Sinapis alba* and *Centaurea cyanus* were counted for each subplot, and weighed on a 4-decimal mass balance. For *Matricaria recutita, Phacelia tanacetifolia*, and *G. segetum*, a subsample of 50 seeds was taken and counted and weighed for each sub-plot, because of the high abundance of seeds per sample. The total amount of seeds per sub-plot was weighed as well, to determine the total number of seeds per sub-plot by dividing the total seed biomass by the biomass of 50 seeds.

The seeds extracted from the plants were used to test whether the F1 generation was affected in its seed germination success. *G. segetum* was not included in the experiment, because the fruits were harvested too late in the season to be included in the germination experiment. This was caused by the later development of the seeds in comparison with the other species. Following Baskin and Baskin (2014), seeds were placed in a dry climate room at ∼2–6°C for 1 month to break seed dormancy. The germination experiment was conducted in a climate room with an air temperature of 20°C, a light intensity of 350 $\mu E/m^2/s$ at seed level and a light/dark period of 16/8 h (Baskin and Baskin, 2014). The seeds were placed in petri-dishes on a layer of filter paper. Fifty seeds per petri-dish were used for *C. cyanus, M. recutita*, and *P. tanacetifolia*, while 30 seeds of *S. alba* were used per dish, because fewer seeds were available. Treatment and control experiments were carried out in triplicate according to the guidelines by Baskin and Baskin (2014). The petri-dishes were placed in a randomized block design and were rotated after each measurement. The seeds were kept moist by adding tap water if necessary. In the first week, the seed germination was recorded each day, while in the subsequent 3 weeks, measurements were performed every 2 days. The total duration of the germination experiment was 4 weeks.

Chemicals

The treatments consisted of one control and five dosages: 0, 0.0097, 0.0193, 0.058, 0.174, and 0.348 gram active ingredient per hectare (g a.i./ha). We used the herbicide solution "Ally©," 60% of which consists of the active ingredient metsulfuron-methyl. The dosage intervals (Table S1) were based on a reference dosage, viz. the Hazardous Dosage at which 95% of the plants are potentially protected (HD_5), available from greenhouse tests (Boutin et al., 2000). This specific HD_5 of 0.058 g a.i./ha and based on 0.01 g a.i./ha is accepted by the US EPA database, based on vegetative endpoints. A factor of 3 was used to determine the other dosages from the reference dosage of 0.058 g a.i./ha (see Supporting Information). The dosages applied were checked by measuring concentrations of metsulfuron-methyl in the spray tank solutions. In the field, a tank sample (volume ∼2 mL) was added to 2 mL acetonitrile. Intended concentrations in the spray tank solutions were expected to be at a level in which they could be measured directly. A detailed description of the preparation of the "Ally©" herbicide solution is included in the Supplementary Information (S.I.). The solutions were applied on July 16, 2015, when the juvenile plants generally had 2–4 leaves, which is the intended time for spraying plants according to the guidelines for NTTP testing (OECD 208, 2006; OECD 227, 2006). A field sprayer was used to spray directly to the field margin by means of downward spraying.

Data Analysis

The statistical analysis mainly consisted of comparing mean values of endpoints in multiple groups (>2). The data analysis followed a step-wise approach. ANOVA with a *post-hoc* Tukey test was performed if the data were normally distributed and

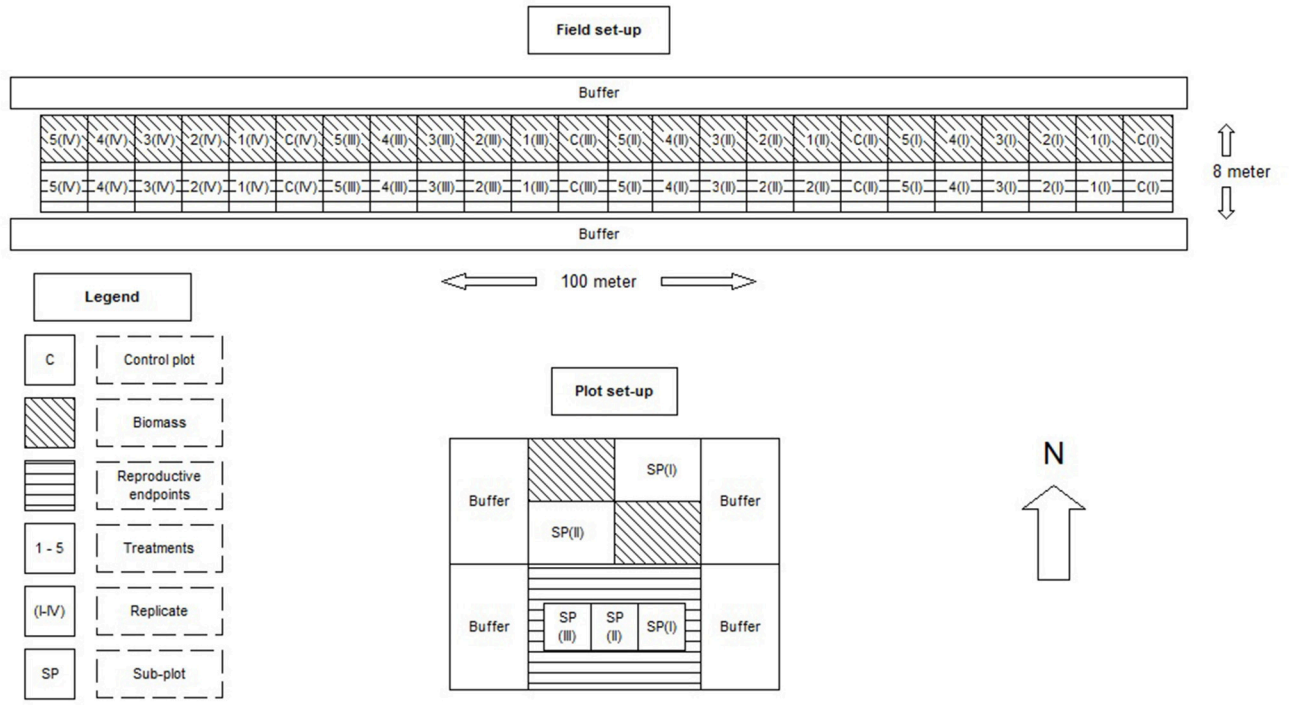


FIGURE 1 | Experimental lay-out of the field margin (above) and the individual plots (below). The treatment levels were: 0, 0.0097, 0.0193, 0.058, 0.174, and 0.348 gram active ingredient per hectare.

if the variances were equal, to test for significant differences between treatments. If data frequencies were not equal between the treatments, the *post-hoc* Scheffe's ANOVA test was used. If the data was not normally distributed, a Ln-transformation or other transformation (Log10 or ArcSin) was first performed. If the data was then still not normally distributed, a Kruskall Wallis test was applied. If variances were not equal, a Welch ANOVA test was performed. Effective Dosages (EDx values) were calculated in GENstat (18th edition). No Observed Effect Dosages (NOEDs; de Snoo et al., 2005) were calculated at parameter or taxon level using the Williams test (Williams, 1972). The analyses were performed with the Community Analysis computer program (Hommen et al., 1994). Minimum Detectable Differences were calculated in percentages (%MDD). The MDDs were categorized into five classes in accordance with EFSA PPR Panel (2013) and Brock et al. (2015). If the MDD exceeds 100%, no effects can be determined statistically. If the MDD is below 50%, small effects can be determined. In between, there are two further classes (<70%; <90%). All other calculations and statistical tests were performed in SPSS (version 22), R and Microsoft Excel.

RESULTS

Verification of the Dosing Solutions

In the spray tank solutions, concentrations of metsulfuron-methyl were found to be in the range of 87.94–97.12% of intended concentrations (Table S2). These results meet the experimental requirements, i.e., measured concentrations are between 80 and 120% of intended concentrations.

Vegetative and Reproductive Endpoints

The herbicide treatments had very little effect on the biomass of all tested plant species, except for *M. recutita*. Significant differences between the treatments were found for this species ($p < 0.05$, Kruskall Wallis test) with an ED50 of 0.06 g a.i./ha (Table S3; **Table 2**) and a NOED of 0.174 g a.i./ha (MDD: 72.02%). For the other species, intraspecific variability was high among the plots. The abundance of individuals, especially *S. alba* and *L. perennis,* was very low (Table S3). A particular plant species might be present in some plots but absent from other plots with the same treatment level.

Reproductive endpoints could only be tested for *S. alba, M. recutita, P. tanacetifolia, C. cyanus,* and *G. segetum*, because these were the only species from which seeds could be collected. *L. perennis* and *M. officinalis* did not produce seeds, as they are perennial plants. Of all the reproductive endpoints we tested (number of seeds and number of fruits, total biomass of seeds and total biomass of fruits, number of seeds per fruit, mass per fruit and mass per seed), the "mass per seed" and "number of seeds per fruit" seemed to be the most sensitive. However, no significant differences were found between the treatments. **Table 2** shows the values of three different reproductive endpoints. The control plots had the highest values of these endpoints in comparison with the plots treated with herbicide. *S. alba* in particular showed a high sensitivity of the "mass per seed" endpoint. *C. cyanus* and

M. reticutata seemed to be mainly affected in their "number of seeds per fruit."

Plant Cover

Figure 2 shows that, in general, the lowest plant covers were found at the highest field dosages (0.174 and 0.348 g a.i./ha), and that the values at the highest dosage differed significantly from those at the control plots after 4 weeks ($p < 0.05$, Tukey's ANOVA test). Furthermore, 14 significant differences ($p < 0.05$, Tukey's ANOVA test) were found between treatment levels of 0, 0.0097, 0.0193 and 0.058 g a.i./ha and the highest dosage (0.348 g a.i./ha). These differences were found in the first 7 weeks after spraying, while after this period only three significant differences ($p < 0.05$, Tukey's ANOVA tests) were found between the lower dosages and the 0.174 g a.i./ha dosage. Additionally, a NOED of 0.058 g a.i./ha was found in weeks 6 and 7 (MDD: 9.69 and 12.73%, respectively; Table S5). During the first 6 weeks of the monitoring period the highest plant cover was not observed in the controls and the lowest dosage of 0.0097 g a.i./ha, but at the 0.0193 g a.i./ha treatment level. Apparently, this low dosage increased plant abundance. Visual assessments showed that there was a tendency toward an increase in grasses (*Poaceae)* in plots with field dosages of 0.174 and 0.348 g a.i./ha, at the cost of the species we had sown in the field margin.

TABLE 2 | Effective Dosages reducing 50% of a specific endpoint (ED50 in g a.i./ha), for several endpoints.

Species	Biomass	Mass per fruit	Mass per seed	Number of seeds per fruit
S. alba	3.51 (–;–)	1.95 (3.07E-3; 1.24E3)	0.02 (2.15E-6; 1.01E2)	–
M. recutita	0.06 (0.008; 0.403)	2.49 (2.88E-13; 1.51E5)	13.7 (–;–)	0.237 (0.15; 0.374)
P. tanacetifolia	–	–	1.01 (7.35E-4; 1.49E3)	–
C. cyanus	1.92 (–;–)	–	0.34 (–;–)	0.007 (3.09E-9; 1.60E4)
L. perennis	–	–	–	–
M. officinalis	0.42 (–;–)	–	–	–
G. segetum	–	1.46 (0.061; 35.096)	–	–

–, No value could be measured and calculated. Confidence intervals shown in parentheses.

Germination Experiment

Figure 3 shows the average germination rates per treatment for *S. alba (a), M. recutita (b), P. tanacetifolia (c),* and *C. cyanus (d)*. Statistical tests (Kruskall Wallis non-parametric test) showed that 3 out of 4 species ($p < 0.05$ for *S. alba, P. tanacetifolia,* and *C. cyanus*) had significant differences in germination percentage at different dosages. The germination of *S. alba* seeds from plots with dosages of 0.058 g a.i./ha and higher was affected. The seed germination rate of *S. alba* was quite low (<30% in controls), while the seeds from the lowest dosage plots (0.0097 and 0.0193 g a.i./ha) showed a relatively high germination rate (50 and 24%, respectively %). The germination rates at 0.058 g a.i./ha and higher are affected (**Figure 3**).

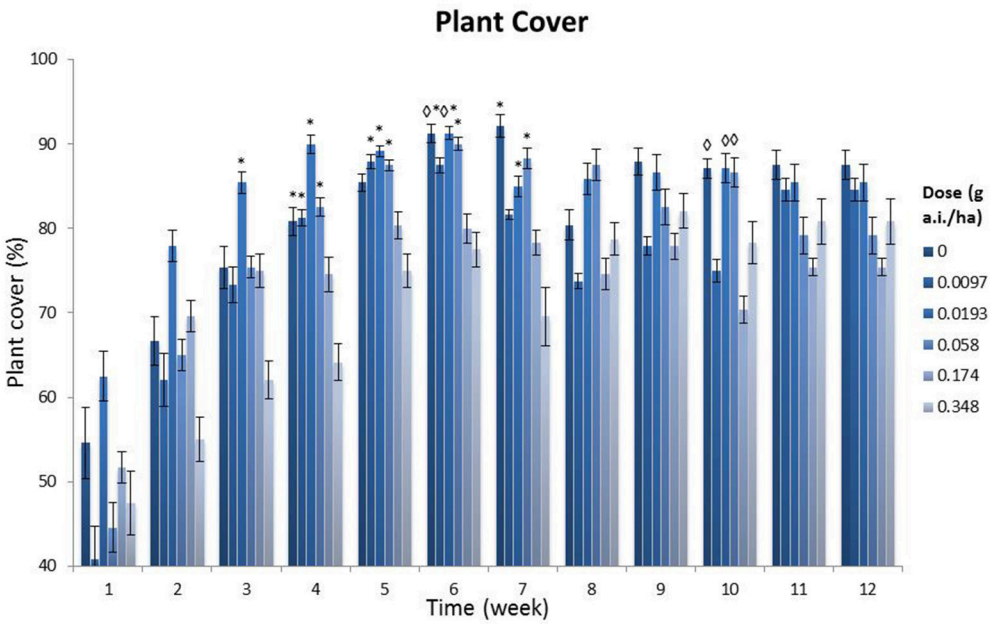


FIGURE 2 | Plant cover per treatment level, showing a time series (weekly monitoring over a total of 12 weeks after spraying). g a.i./ha: gram active ingredient per hectare. The error bars represent the standard error. *Significant difference with the 0.348 g a.i./ha dosage ($p < 0.05$, one-way Tukey's ANOVA). ◊Significant difference with the 0.174 g a.i./ha dosage ($p < 0.05$, one-way Tukey's ANOVA).

M. recutita showed a high germination rate (100% in controls). The germination rate of the seeds of *M. recutita* was the highest of all species (71.1% in the controls). Although no direct trend was found between the dosages (**Figure 3**), we found an NOED of 0.058 g a.i./ha (MDD: 39%; Table S4). For *P. tanacetifolia* and *C. cyanus*, there was a reduction in the germination of seeds from plots with dosages of 0.058 and 0.0193 g a.i./ha, respectively (MDD: 78 and 69% respectively) and higher (Table S4). However, the seed germination was very low for both species (14% in controls for *P. tanacetifolia* and 6% in controls for *C. cyanus*). Hormesis seemed to occur in *S. alba, P. tanacetifolia* and *C. cyanus*, implying that relatively low dosages of metsulfuron-methyl (0.0097 and 0.0193 g a.i./ha) stimulated seed germination.

DISCUSSION

Discussion of Results

The results show that biomass was not affected by the herbicide for any of the species except *M. recutita*. On the other hand, several other vegetative plant endpoints (width, height etc.) could be potentially affected, as metsulfuron-methyl limits cell division (Russell et al., 2002). This can limit the size of the plant, without the biomass being affected. However, we did not measure these endpoints. We found no significant differences between the treatments regarding the reproductive endpoints. However, the data suggests that the values of "mass per seed" and "number of seeds per fruits" in particular decreased with increasing dosages. The lack of statistical differences can be explained by the low number of observations, which also varied between the plots, reducing the power of the statistical tests. Other studies have demonstrated that reproductive endpoints are indeed very sensitive to metsulfuron-methyl (Boutin et al., 2000; Blackburn and Boutin, 2003; Olszyk et al., 2004; Kjær et al., 2006; Carpenter and Boutin, 2010; Pfleeger et al., 2012; Schmitz et al., 2014, 2015). Our results also showed hormesis occurring in *S. alba*, *P. tanacetifolia* and *C. cyanus*, at low dosages of metsulfuron-methyl and in both vegetative and reproductive endpoints. Hormesis is a phenomenon often observed in plant studies (Duke et al., 2006).

Our results showed that plant cover differed significantly between the highest dosage (0.348 g a.i./ha) on the one hand and the control and other treatment levels on the other hand. Fourteen significant differences were found in the first 7 weeks after the herbicide exposure. Thereafter, only in 1 week were three significant differences found between treatments. In addition, an NOED of 0.058 g a.i./ha was found in weeks 6 and 7, while an NOED of 0.348 g a.i./ha was found in week 8. This difference suggests that plants can recover from spraying, as was also found in other studies (de Snoo et al., 2005; Carpenter and Boutin, 2010; Strandberg et al., 2012; Schmitz et al., 2014). In comparison with lower-level treatments, plots with dosages of 0.174 and 0.348 g a.i./ha featured more grasses (*Poaceae*), as we found through visual observation. Grasses are not a direct target of the herbicide (Russell et al., 2002) and are more resistant to dosages of 0.174 and 0.348 g a.i./ha. Grasses in these high dosage plots may have contributed to the recovery of the plant cover, instead of the species we sowed.

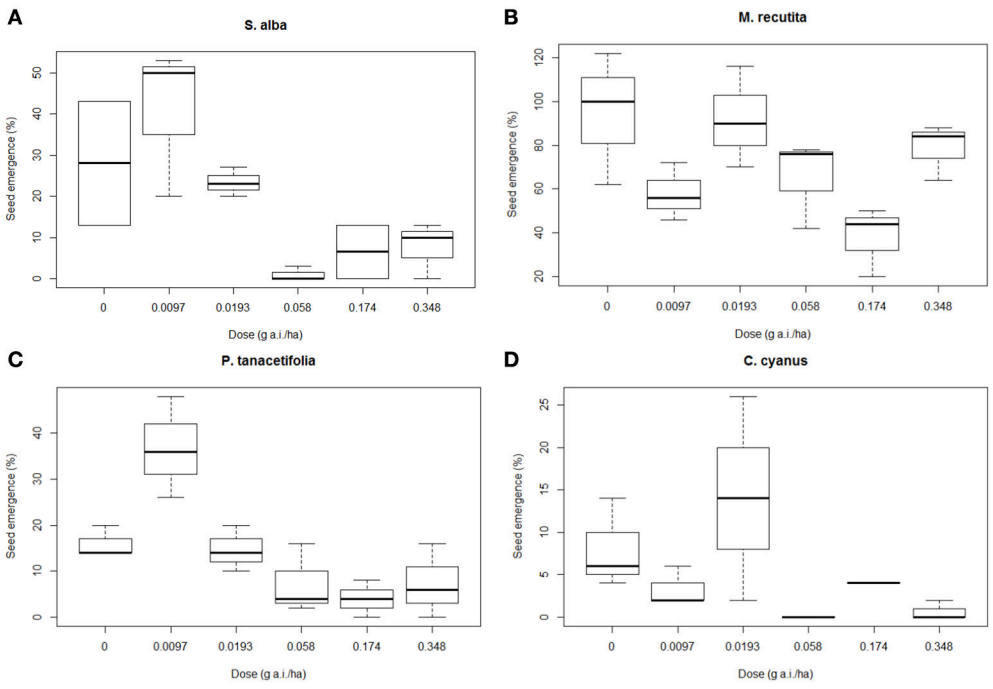


FIGURE 3 | Boxplots presenting the means of germination succes for each species and each treatment level, as a percentage of the total number of seeds per replicate. (A) *Sinapis alba*, **(B)** *Matricaria recutita*, **(C)** *Phacelia tanacetifolia,* and **(D)** *Centaurea cyanus.* g a.i./ha: gram active ingredient per hectare.

The statistically significantly lower seed germination at the higher herbicide dosages can thus potentially lead to shifts in species composition and succession of the vegetation (Schmitz et al., 2014), with higher frequencies of tolerant species (Geiger et al., 2010). For example, *S. alba* could disappear from field margins next to arable fields in the long run, because of reduced seed quality (such as seed biomass and seed germination success) at dosages of 0.0193 g a.i./ha or higher at a normal field application dosage of 0.058 g a.i./ha. In addition, *P. tanacetifolia* and *C. cyanus* also showed significantly reduced germination of seeds at higher field dosages (higher than 0.0193 or 0.058 g a.i./ha). In the long term, this could lead to a situation where these susceptible species disappear and tolerant species (such as *M. recutita* in the present experiment, based on the germination results) gain a greater abundancy. However, the biomass of this species was highly affected by the herbicide. This indicates that the effects of metsulfuron-methyl are complex, because there is no single endpoint which is the most sensitive for all species. Hence, this study shows that the accepted HD_5 of 0.058 g a.i./ha is not protective in terms of plant cover, and especially in terms of F1 seed germination success, as the NOED was 0.058 g a.i./ha or lower.

In the present study, the difference between the biomass and reproductive endpoints at various dosages, based on the NOEDs, was minor. However, plant cover and seed germination rates had much lower NOEDs (0.058 g a.i./ha and lower). This indicates that these endpoints were the most sensitive ones in this field study. The vegetative and reproductive endpoints tested in this field trial showed a high variability, which limited clear dose-response results. Apparently, it is not only the herbicide treatment which influenced the effects, but also many co-variables. Several possible factors could be mentioned. First, the test duration was restricted to one growing season. More data based on multiple years would give a better estimate of the mean effect, as the field study by Schmitz et al. (2014) revealed, where the effects of a herbicide became stronger over time. At the time when we sowed the seeds, there was a severe drought lasting several weeks, which could have influenced the germination success of the seeds. Testing across multiple years would average out these (extreme) weather conditions. Second, some individuals were exposed directly, while other individuals of the same species had not germinated yet at the moment of spraying. However, the herbicide is mobile in the soil (Blair and Martin, 1988; Russell et al., 2002), so the herbicide could have been taken up by both foliage and roots and transported via xylem and phloem later on (Blair and Martin, 1988). Third, the present study used a realistic field margin set-up with a seed mixture consisting of multiple species, and additional species developed in the field. This could have resulted in species interactions and competition, leading to different abundances per species and plot. However, we were not able to find any statistically significant interactions between species in terms of plant abundances, population dynamics or succession.

Recommendations

The present paper has focused on the germination experiment and some results regarding vegetative and reproductive endpoints. However, this study also intended to provide information and recommendations for further experiments with NTTPS and herbicides in real field settings. The most important recommendations in this respect are listed in **Table 3**.

CONCLUSION

In the introduction, the following questions were raised: (1) How does the sensitivity of reproductive endpoints differ from that of vegetative endpoints? (2) Is there an effect on the next generation of herbicide-exposed NTTPs? (3) How do species differ in their sensitivity to the herbicide? Related to question 1, this paper has shown that reproductive endpoints, especially "mass per seed" and "number of seeds per fruit," seemed to be more sensitive than vegetative endpoints. Only the biomass of *M. recutita* was affected at the treatment levels we tested (0.058 g a.i./ha and higher). Field dosages of 0.174 and 0.348 g a.i./ha led to significantly different plant cover values compared to lower dosages and controls. The answer to question 2, i.e., are there any effects on the next generation of herbicide-exposed NTTPs, was shown by studying the seed germination of the F1 generation. Some species were affected at relatively low dosages of the sulfonyl urea herbicide metsulfuron-methyl. The results of the germination experiment to test whether the herbicide had an impact on the next generation showed that three out of four species (*S. alba, P. tanacetifolia,* and *C. cyanus)* had significantly lower seed germination rates at herbicide dosages of 0.0193 g a.i./ha and higher. The third question that is related to the differences in sensitivity between species, can be confirmed. The plant species involved reacted differently to the herbicide. *S. alba* was highly affected in terms of reproductive endpoints and seed germination, while biomass proved more sensitive in

TABLE 3 | Recommendations for further studies.

Type of subject	Recommendation
Plant species	(1) Select species with seeds which are easy to process (>0.15 mg per seed) to reduce human-made errors. (2) Include both annual and perennial species.
Experimental set-up	(1) If time resources are very limiting, one can decide to only include reproductive endpoints instead of both reproductive and vegetative endpoints. (2) Test duration must be at least two growing seasons to investigate the total herbicide effect. In this case, perennial species can be included as well for the reproductive analysis, at least in the second growing season. (3) Include a germination experiment with seeds obtained from herbicide-exposed plants. (4) Always include reproductive endpoints in this type of ecological risk assessment. Biomass as a vegetative endpoint can be omitted for this Mode of Action, as width, height etc. of plants are better vegetative endpoints to assess the effects of sulfonylureas.
Measurements	(1) Harvest fruits and seeds. (2) Monitor reproductive endpoints, estimated cover and plant abundance weekly. Double the monitoring frequencies during the first 2 weeks to include juvenile plants.

M. recutita. Reproductive endpoints and seed germination were more sensitive in *P. tanacetifolia* and *C. cyanus*. We recommend that plant reproductive endpoints and germination experiments are included in this type of risk assessment to investigate the total herbicide effect on the full life cycle and population fitness of the plants. Currently, effects on reproduction are not included in standard guidelines.

AUTHOR CONTRIBUTIONS

JN performed the field research for his master, provided the manuscript, performed the data analyses, and literature research. RW participated in the field work and contributed to writing and critically reviewed the manuscript. IR organized the pesticide dosing of the field margin, performed part of the statistical analyses and critically reviewed the manuscript. GA was supervisor of JN and had the final responsibility for the manuscript, the editing and the submission.

FUNDING

The research was part of a master of Wageningen University and Research.

ACKNOWLEDGMENTS

We would like to thank A.M. (Arrienne) Matser for her valuable contribution by preparing the spray solutions. In addition, we appreciate the work of C.H.F.M (Henri) JN, who measured several endpoints and assisted in setting up the germination experiment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2017.00010/full#supplementary-material

REFERENCES

Arts, G. H. P., Dollinger, M., Kohlschmid, E., Maltby, L., Ocho-Acuna, H., and Poulsen, V. (2015). An ecosystem services approach to pesticide risk assessment and risk management of non-target terrestrial plants: recommendations from a SETAC Europe workshop. *Environ. Sci. Pollut. Res. Int.* 22, 2350–2355. doi: 10.1007/s11356-014-3637-6

Baskin, C. C., and Baskin, J. M. (2014). *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. San Diego, CA: Academic Press.

Blackburn, L. G., and Boutin, C. (2003). Subtle effects of herbicide use in the context of genetically modified crops: a case study with Glyphosate (Roundup®). *Ecotoxicology* 12, 271–285. doi: 10.1023/A:1022515129526

Blair, A. M., and Martin, T. D. (1988). A review of the activity, fate and mode of action of sulfonylurea herbicides. *Pest Manage. Sci.* 22, 195–219. doi: 10.1002/ps.2780220303

Boutin, C., Aya, K. L., Carpenter, D., Thomas, P. J., and Rowland, O. (2012). Phytotoxicity testing for herbicide regulation: shortcomings in relation to biodiversity and ecosystem services in agrarian systems. *Sci. Total Environ.* 415, 79–92. doi: 10.1016/j.scitotenv.2011.04.046

Boutin, C., Elmegaard, N., and Kjaer, C. (2004). Toxicity testing of fifteen non-crop plant species with six herbicides in a greenhouse experiment: implications for risk assessment. *Ecotoxicology* 13, 349–369. doi: 10.1023/B:ECTX.0000033092.82507.f3

Boutin, C., Lee, H. B., Peart, E. T., Batchelor, P. S., and Maguire, R. J. (2000). Effects of the sulfonylurea herbicide metsulfuron methyl on growth and reproduction of five wetland and terrestrial plant species. *Environ. Toxicol. Chem.* 19, 2532–2541. doi: 10.1002/etc.5620191020

Boutin, C., and Rogers, C. A. (2000). Pattern of sensitivity of plant species to various herbicides—an analysis with two databases. *Ecotoxicology* 9, 255–272. doi: 10.1023/A:1026518027350

Boutin, C., Strandberg, B., Carpenter, D., Mathiassen, S. K., and Thomas, P. J. (2014). Herbicide impact on non-target plant reproduction: what are the toxicological and ecological implications? *Environ. Pollut.* 185, 295–306. doi: 10.1016/j.envpol.2013.10.009

Brock, T. C. M., Hammers-Wirtz, M., Hommen, U., Preuss, T. G., Ratte, H. T., Roessink, I., et al. (2015). The Minimum Detectable Difference (MDD) and the interpretation of treatment-related effects of pesticides in experimental ecosystems. *Environ. Sci. Pollut. Res.* 22, 1160–1174. doi: 10.1007/s11356-014-3398-2

Carpenter, D., and Boutin, C. (2010). Sublethal effects of the herbicide glufosinate ammonium on crops and wild plants: short-term effects compared to vegetative recovery and plant reproduction. *Ecotoxicology* 19, 1322–1336. doi: 10.1007/s10646-010-0519-7

de Snoo, G. R., Tamis, W. L. M., and van de Brink, P. J. (2005). *Non Target Plant Field Study: Effects of Glufosinate-Ammonium on Off Crop Vegetation*. CML report 161, Department of Environmental Biology, 114.

Duke, S. O., Cedergreen, N., Velini, E. D., and Belz, R. G. (2006). Hormesis: is it an important factor in herbicide use and allelopathy? *Outlooks Pest Manage.* 17, 29–33. doi: 10.1564/16feb10

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues) (2014). Scientific Opinion addressing the state of the science on risk assessment of plant protection products for non-target terrestrial plants. *EFSA J.* 12:3800. doi: 10.2903/j.efsa.2014.3800

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues) (2013). Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. *EFSA J.* 11:3290. doi: 10.2903/j.efsa.2013.3290

Geiger, F., Bengtsson, J., Berendse, F., Weisser, W. W., Emmerson, M., Morales, M. B., et al. (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic Appl. Ecol.* 11, 97–105. doi: 10.1016/j.baae.2009.12.001

Hommen, U., Dülmer, U., Veith, D. (1994). "A computer program to evaluate plankton data from freshwater field tests," in *Freshwater Field Tests for Hazard Assessment of Chemicals,* eds I. A, Hill, F. Heimbach, P. Leeuwangh, and P. Matthiesen (Boca Raton, FL: Lewis Publishers), 503–513.

Kjær, C., Strandberg, M., and Erlandsen, M. (2006). Metsulfuron spray drift reduces fruit yield of hawthorn (*Crataegus monogyna* L.). *Sci. Total Environ.* 356, 228–234. doi: 10.1016/j.scitotenv.2005.03.019

OECD 208 (2006). *GUIDELINES FOR THE TESTING OF CHEMICHALS. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*. Paris: OECD.

OECD 227 (2006). *GUIDELINES FOR THE TESTING OF CHEMICHALS. Terrestrial Plant Test: Vegetative Vigour Test*. Paris: OECD.

Olszyk, D. M., Burdick, C. A., Pfleeger, T. G., lee, E. H., and Watrud, L. S. (2004). Assessing the risks to non-target terrestrial plants from herbicides. *J. Agric. Meteorol.* 60, 221–242. doi: 10.2480/agrmet.60.221

Pfleeger, T., Blakeley-Smith, M., King, G., Lee, E. H., Plocher, M., and Olszyk, D. (2012). The effects of glyphosate and aminopyralid on a multi-species plant field trial. *Ecotoxicology* 21, 1771–1787. doi: 10.1007/s10646-012-0912-5

Robinson, R. A., and Sutherland, W. J. (2002). Post-war changes in arable farming and biodiversity in Great Britain. *J. Appl. Ecol.* 39, 157–176. doi: 10.1046/j.1365-2664.2002.00695.x

Rokich, D. P., Harma, J., Turner, S. R., Sadler, R. J., and Tan, B. H. (2009). Fluazifo-p-p-butyl herbicide: implications for germination, emergence and growth of Australian plant species. *Biol. Conserv.* 142, 850–869. doi: 10.1016/j.biocon.2008.12.013

Russell, M. H., Saladini, J. L., and Lichtner, F. (2002). Sulfonylurea herbicides. *Pesticide Outlook* 13, 166–173. doi: 10.1039/b206509f

Schmitz, J., P., Stahlschmidt, C. A., Brühl (2015). *Protection of Terrestrial Non-Target Plant Species in the Regulation of Environmental Risks of Pesticides.* Umweltbundesamt.

Schmitz, J., Schäfer, K., and Brühl, C. A. (2014). Agrochemicals in field margins—field evaluation of plant reproduction effects. *Agric. Ecosyst. Environ.* 189, 82–91. doi: 10.1016/j.agee.2014.03.007

Strandberg, B., Bruus, M., Kjær, C., Damgaard, C., Andersen, H. V., Bossi, R., et al. (2012). *Effects of Herbicides on Non-Target Plants: How Do Effects in Standard Plant Tests Relate to Effects in Natural Habitats*? Miljøstyrelsen.

Williams, D. A. (1972). The comparison of several dose levels with zero dose control. *Biometrics* 28, 519–531.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Nelemans, van Wijngaarden, Roessink and Arts. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 20 June 2017
doi: 10.3389/fenvs.2017.00034

Glyphosate-Induced Specific and Widespread Perturbations in the Metabolome of Soil *Pseudomonas* Species

Ludmilla Aristilde[1,2,3]*, *Michael L. Reed*[1], *Rebecca A. Wilkes*[1], *Tracy Youngster*[2], *Matthew A. Kukurugya*[1], *Valerie Katz*[1] *and Clayton R. S. Sasaki*[1]

[1] *Department of Biological and Environmental Engineering, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY, United States,* [2] *Soil and Crop Sciences Section, School of Integrative Plant Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY, United States,* [3] *Atkinson Center for a Sustainable Future, Cornell University, Ithaca, NY, United States*

OPEN ACCESS

Edited by:
Johann G. Zaller, University of Natural Resources and Life Sciences, Vienna, Austria

Reviewed by:
Robert J. Kremer, University of Missouri, United States
Marcelo Pedrosa Gomes, Universidade Federal de Minas Gerais, Brazil

***Correspondence:**
Ludmilla Aristilde
ludmilla@cornell.edu

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *13 March 2017*
Accepted: *06 June 2017*
Published: *20 June 2017*

Citation:
Aristilde L, Reed ML, Wilkes RA, Youngster T, Kukurugya MA, Katz V and Sasaki CRS (2017) Glyphosate-Induced Specific and Widespread Perturbations in the Metabolome of Soil Pseudomonas Species. Front. Environ. Sci. 5:34. doi: 10.3389/fenvs.2017.00034

Previous studies have reported adverse effects of glyphosate on crop-beneficial soil bacterial species, including several soil *Pseudomonas* species. Of particular interest is the elucidation of the metabolic consequences of glyphosate toxicity in these species. Here we investigated the growth and metabolic responses of soil *Pseudomonas* species grown on succinate, a common root exudate, and glyphosate at different concentrations. We conducted our experiments with one agricultural soil isolate, *P. fluorescens* RA12, and three model species, *P. putida* KT2440, *P. putida* S12, and *P. protegens* Pf-5. Our results demonstrated both species- and strain-dependent growth responses to glyphosate. Following exposure to a range of glyphosate concentrations (up to 5 mM), the growth rate of both *P. protegens* Pf-5 and *P. fluorescens* RA12 remained unchanged whereas the two *P. putida* strains exhibited from 0 to 100% growth inhibition. We employed a ^{13}C-assisted metabolomics approach using liquid chromatography-mass spectrometry to monitor disruptions in metabolic homeostasis and fluxes. Profiling of the whole-cell metabolome captured deviations in metabolite levels involved in the tricarboxylic acid cycle, ribonucleotide biosynthesis, and protein biosynthesis. Altered metabolite levels specifically in the biosynthetic pathway of aromatic amino acids (AAs), the target of toxicity for glyphosate in plants, implied the same toxicity target in the soil bacterium. Kinetic flux experiments with ^{13}C-labeled succinate revealed that biosynthetic fluxes of the aromatic AAs were not inhibited in *P. fluorescens* Pf-5 in the presence of low and high glyphosate doses but these fluxes were inhibited by up to 60% in *P. putida* KT2440, even at sub-lethal glyphosate exposure. Notably, the greatest inhibition was found for the aromatic AA tryptophan, an important precursor to secondary metabolites. When the growth medium was supplemented with aromatic AAs, *P. putida* S12 exposed to a lethal dose of glyphosate completely recovered in terms of both growth rate and selected

metabolite levels. Collectively, our findings led us to conclude that the glyphosate-induced specific disruption of *de novo* biosynthesis of aromatic AAs accompanied by widespread metabolic disruptions was responsible for dose-dependent adverse effects of glyphosate on sensitive soil *Pseudomonas* species.

Keywords: metabolomics of glyphosate effects, soil *Pseudomonas* species, aromatic amino acid biosynthesis, disruption of cellular metabolome, non-targeted effects

INTRODUCTION

Glyphosate-based herbicides, including notably Roundup®, are the most widely used herbicides in the United States (Woodburn, 2000). This is largely due to the advent of Roundup Ready crops, in which the gene that encodes the enzymatic target of the active ingredient glyphosate is modified (Duke et al., 2012) (**Figure 1A**). Thus, these genetically-modified crops (e.g., soybean, corn) are protected against glyphosate applications. Glyphosate is lethal to targeted weed plants by inhibiting 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an essential enzyme in the shikimate pathway that is responsible for the biosynthesis of aromatic amino acids (AAs) (Schulz et al., 1985) (**Figure 1B**). Although mammalian organisms including humans do not synthesize aromatic AAs and instead obtain these AAs from their diet, microorganisms employ the shikimate pathway similar to plants to synthesize their aromatic AAs. Therefore, of emerging concern are the potential non-targeted effects of glyphosate on soil microorganisms that are important to soil and crop health.

There have been numerous reports of adverse effects of glyphosate treatments on various microbial species and communities (Lévesque et al., 1987; Larson et al., 2006; Kremer and Means, 2009; Zobiole et al., 2011; Lane et al., 2012; Druille et al., 2013, 2015). Here we seek to gain insights into the intracellular targets underlying the response of soil *Pseudomonas* species, such as *P. putida, P. fluorescens,* and *P. protegens*, to glyphosate exposure. Due to their production of a range of antibiotic compounds, iron-scavenging molecules (or siderophores), and plant growth promoters, several *Pseudomonas* species have been employed as effective biocontrol agents to protect plants against pathogens and promote plant health (Timmis, 2002). A decreased abundance in *Pseudomonas* species was reported in greenhouse soils wherein glyphosate was applied to soybeans in a manner similar to agricultural applications (Zobiole et al., 2011). Furthermore, a previous study revealed greater inhibition of the EPSP synthase activity in *P. fluorescens* AFT36 than in *P. putida* W1616 at the same glyphosate concentration (Schulz et al., 1985). We hypothesize that the sensitivity of glyphosate-exposed *Pseudomonas* species in glyphosate-containing soils could be due to (1) species-specific growth responses to glyphosate exposure, (2) glyphosate inhibition of the biosynthetic pathway for aromatic AAs, or (3) disruption of other cellular pathways.

Species-specific bacterial growth response to glyphosate have been reported previously (Santos and Flores, 1995; Lancaster et al., 2010; Duke et al., 2012). The glyphosate concentration required for 50% growth inhibition of *Escherichia coli*, *Bacillus subtilis*, *B. jabonicum*, and *P. aeruginosa*, was estimated to be, respectively, 75 μM, 174 μM, 1.1 mM, and 1.1 mM (Duke et al., 2012). Differences in adverse metabolic effects were also reported for two closely-related nitrogen-fixing bacteria, *Azotobacter chroococcum* and *A. vinelandii*, in response to similar glyphosate exposure (Santos and Flores, 1995). Specifically, at 4 kg ha^{-1} of glyphosate, the nitrogenase activity in *A. vinelandii* was decreased by 22% but only by 2% in *A. chroococcum* (Santos and Flores, 1995). At 3 times higher glyphosate application (12 kg ha^{-1}), the reduction in nitrogenase activity was 45 and 13%, respectively, in *A. vinelandii* and *A. chroococcum* (Santos and Flores, 1995). Furthermore, a decrease in the cell size of the glyphosate-exposed *Azotobacter* species was proposed to be a result of a reduction in protein synthesis downstream of EPSP inhibition (Santos and Flores, 1995). Whether the decrease in *Pseudomonas* species in glyphosate-exposed soils is due to a similar metabolic disruption remains to be determined.

Species-dependent differences in the EPSP synthase are at the basis of the generation of Roundup Ready crops. In these crops, a bacterial gene from *Agrobacterium* sp. strain CP4 is inserted into the crops in order to produce an EPSP synthase resistant to glyphosate (Funke et al., 2006). Thus, the primary targets of glyphosate-based herbicides are plants with glyphosate-sensitive EPSP synthase in their shikimate pathway. However, in addition to plants, the shikimate pathway is responsible for the *de novo* biosynthesis of aromatic AAs in microorganisms (**Figure 1B**). In this pathway, shikimate is eventually converted to chorismate, the common metabolite precursor to the following three aromatic amino AAs: tyrosine, phenylalanine, and tryptophan (**Figure 1B**). The direct precursor to chorismate, 5-enolpyruvylshikimate-3P, is synthesized by the EPSP enzyme. Therefore, the EPSP synthase represents a gateway to the production of aromatic AAs (**Figure 1B**). Glyphosate interferes with this gateway by competing with the endogenous substrate, phosphoenolpyruvate (PEP) (**Figure 1C**), which combines with shikimate-3-phosphate to produce EPSP (Steinrücken and Amrhein, 1980; Schönbrunn et al., 2001). Different extents of inhibition of EPSP synthase were reported previously for different *Pseudomonas* species (Schulz et al., 1985). Specifically, at about 3.0 μM glyphosate, there was 50% inhibition of the enzyme activity in *P. fluorescens* AFT36 but no inhibition in *P. putida* W1616 (Schulz et al., 1985). At one order of magnitude higher concentration (3 mM) of glyphosate, there was 100% inhibition of EPSP synthase in *P. fluorescens* AFT36 but only 50% inhibition in *P. putida* W1616 (Schulz et al., 1985). It has not been demonstrated, however, explicitly that the inhibition of the EPSP synthase resulted in the inhibition of the biosynthesis of aromatic AAs in these *Pseudomonas*

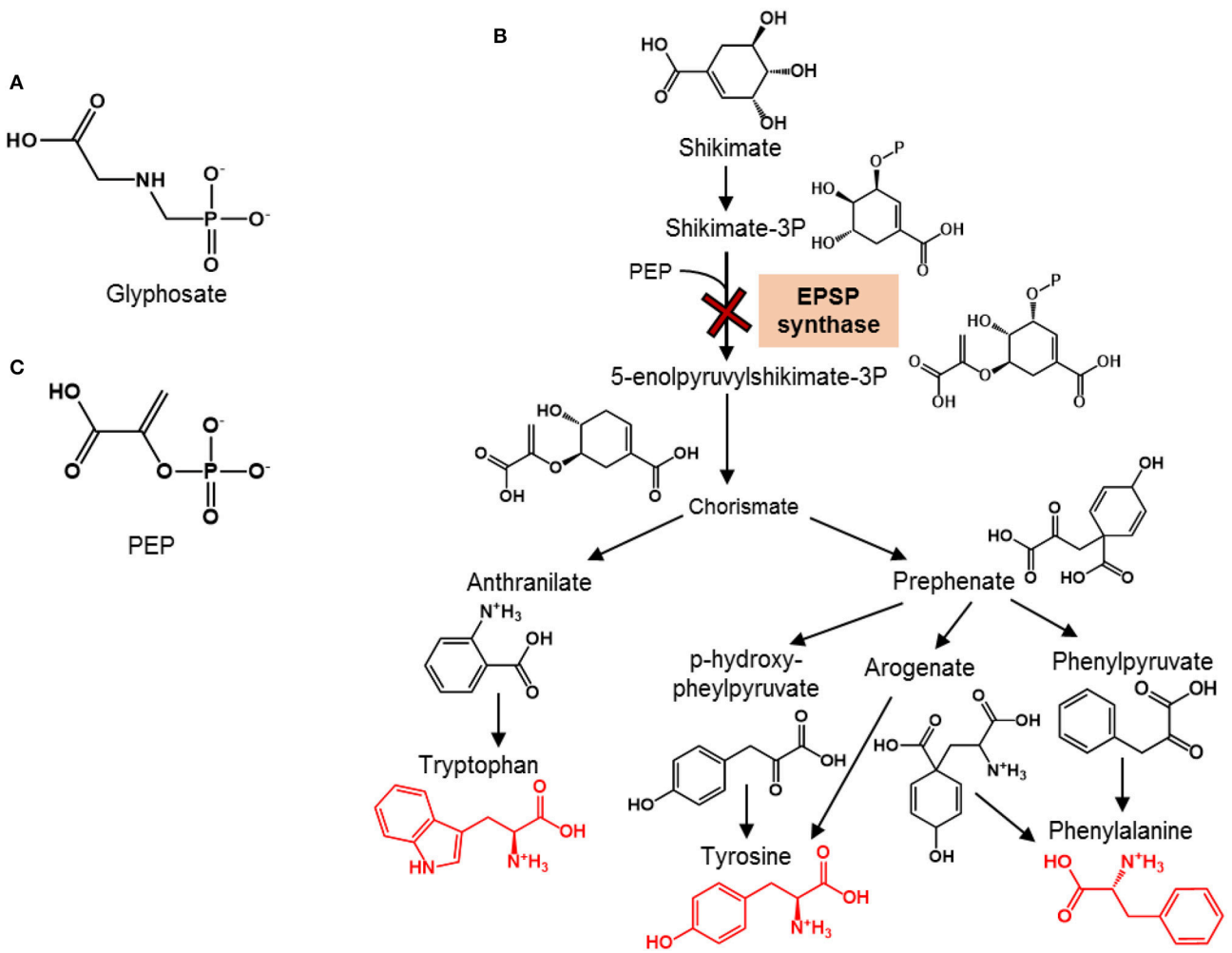


FIGURE 1 | Chemical structures of glyphosate and phosphoenolpyruvate and overview of the shikimate pathway for aromatic amino acid synthesis. **(A)** Chemical structure of the herbicide glyphosate. **(B)** Schematic of the shikimate pathway for the biosynthesis of aromatic amino acids (shown in red structures) in plants (adapted from Tzin and Galili, 2010); the toxicity target of glyphosate, which is the enzyme responsible for the synthesis of 5-enolpyruvylshikimate-3-phosphate by combining shikimate-3P and phosphoenolpyruvate (PEP), is shown with the red cross. **(C)** Chemical structure of PEP, the endogenous substrate at the enzymatic target of glyphosate.

species or other crop-beneficial bacterial species susceptible to glyphosate.

A decreased abundance in fungal populations was also reported in soil microbial communities exposed to glyphosate (Johal and Rahe, 1984; Lévesque and Rahe, 1992; Kremer et al., 2005). Stunted root growth of plants in response to increasing glyphosate applications was accompanied by both an increase in the fungal pathogens *Fusarium* spp. and a decrease in *Pseudomonas* spp. (Zobiole et al., 2011). Interestingly, certain soil *Pseudomonas* species are known to produce pyrrolnitrin, an important potent antifungal metabolite, that requires the aromatic AA tryptophan as a metabolite precursor (Kirner et al., 1998). Whether the change in the relative abundance of *Fusarium* spp. and *Pseudomonas* spp. is due to disruption in the biosynthetic pathway that feeds the production of pyrrolnitrin was not determined. In addition to fungicides, crop-beneficial *Pseudomonas* spp. are known to produce auxin-like plant hormones including notably indole-acetic acid (IAA). Similar to pyrrolnitrin, IAA is derived from the aromatic AA tryptophan (Zhao, 2012). In fact, decreased abundance in *Pseudomonas* spp. was found to be positively correlated with reduced populations of IAA-producing bacteria in a glyphosate-treated soil (Zobiole et al., 2011). Due to the important connection of shikimate pathway to the biosynthesis of both primary and secondary metabolites in *Pseudomonas* spp., elucidating the effects of glyphosate on both the initiation of this metabolic pathway as well as its associated synthesis of aromatic AAs is warranted.

In the present study, we evaluated our stated hypotheses regarding the factors responsible for the adverse effects of glyphosate exposure on four different soil *Pseudomonas* species by (1) determining the growth response of these different species to different glyphosate concentrations, (2) evaluating the

biosynthesis of aromatic AAs in the shikimate pathway as a specific target of glyphosate, and (3) investigating other cellular pathways susceptible to glyphosate exposure. We achieved our objectives by employing a metabolomics approach involving liquid chromatography-mass spectrometry (LC-MS) and kinetic flux profiling using stable isotope tracers. Our results provide metabolic insights into both specific and widespread metabolic perturbations of glyphosate to a range of soil *Pseudomonas* species. These findings have broader agricultural relevance in regards to the assessment of potential non-targeted effects of glyphosate to sensitive soil bacterial species.

EXPERIMENTAL METHODS

Culturing Conditions

P. putida KT2440, *P. putida* S12, and *P. protegens* Pf-5 were obtained from ATCC (American Type Culture Collection, Manassas, VA); the agricultural soil isolate *P. fluorescens* (strain RA12) was a gift from Dr. Rania Abou-Kandil (Cornell University). Cell cultures (three biological replicates) were grown at 30°C in a G24 environmental incubator shaker (New Brunswick Scientific, Edison, NJ) at 220 rpm (Sasnow et al., 2016). The pH-adjusted (pH 7.0) and filter-sterilized (0.22 μm nylon; Waters Corporation, Massachusetts) growth medium contained both major and minor nutrient salts: 89.4 mM K_2HPO_4, 56.4 mM NaH_2PO_4, 0.81 mM $MgSO_4{\cdot}7H_2O$, 18.7 mM NH_4Cl, 8.6 mM NaCl, 34 μM $CaCl_2{\cdot}2H_2O$, 30 μM $FeSO_4{\cdot}7H_2O$, 0.86 μM $CuSO_4{\cdot}5H_2O$, 1.9 μM H_3BO_3, 7.7 μM $ZnSO_4{\cdot}7H_2O$, 0.75 μM $MnSO_4{\cdot}5H_2O$, 0.26 μM $NiCl_2{\cdot}6H_2O$, and 0.31 μM $Na_2MoO_4{\cdot}5H_2O$. Additionally, succinate (50 mM) was provided as the source of organic carbon. For glyphosate dosage, the succinate-containing growth medium was prepared with different concentrations of glyphosate (0, 0.5, or 5 mM glyphosate). The choice of glyphosate concentration use here was based on the concentration range used in previous studies on the effects of glyphosate exposure on bacterial species: 0.003–3.0 mM (Schulz et al., 1985); 0.1 mM (Liu et al., 1991); 0.03–10 mM (Forlani et al., 2008); 0.075–1.1 mM (Duke et al., 2012); 0.44–29 mM (Shehata et al., 2013). To determine the influence of aromatic AA supplementation on glyphosate effects on growth, cells were also grown on the same media recipe described above supplemented with a cocktail (0.5 mM each) of the three aromatic AAs (tryptophan, tyrosine, and phenylalanine). All the chemicals listed above were obtained analytical grade from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburg, PA, USA). For all the different growth conditions, the cells were transferred twice into fresh growth medium in order to ensure that cells were fully acclimated in their respective growth medium. Cell growth (three biological replicates) was monitored by measuring the optical density at 600 nm (OD_{600}) using an Agilent Cary UV-visible spectrophotometer (Santa Clara, California).

Sampling Intracellular Metabolite Levels

Intracellular metabolite levels were determined from cell suspensions obtained during exponential growth phase. The suspensions were filtered and the cell-containing filters were immediately quenched by submerging them in a cold (4°C) 2-mL solution of methanol:acetonitrile:water (40:40:20). Solutions with the lysed cells were subsequently filter-centrifuged (Sigma-Aldrich Spin-X 0.22 μm filters). Aliquots of the supernatants were dried under nitrogen gas and re-suspended in LC-MS ultrapure water (Fisher Scientific, Pittsburgh, Pennsylvania) before analysis via LC-MS. Metabolite levels were normalized to biomass quantity at the time of sampling.

Kinetic Intracellular Metabolite Labeling

To capture the influence of glyphosate on *in vivo* metabolic fluxes, we carried out a kinetic flux experiment. This experimental procedure is described in detail elsewhere (Aristilde, 2017). Briefly, cells were first grown in a succinate-containing growth medium recipe as described above. At early exponential phase after the second transfer, aliquots (3 mL) of the cell suspensions were filtered and the cell-containing filters were transferred to a plate with agar-solidified growth medium with unlabeled succinate. Once the cells growing on the plates had reached mid-exponential phase (OD_{600} 0.5–1.0) as described previously by Aristilde (2017), the cell-containing filters were subsequently transferred to plates that contained labeled [U-$^{13}C_4$]-succinate (50 mM) with 0, 0.5 or 5 mM glyphosate. At specific time points (5, 12, and 30 min) following the isotopic switch, the cell-containing filters were quenched and prepared as described above. Control experiments for the 0-min time point were conducted using cell-containing filters from plates with unlabeled succinate. The labeled substrate was obtained from Cambridge Isotopes (Tewskbury, MA, USA).

Metabolomics Analysis via LC-MS

The metabolite extracts were analyzed by reversed-phase ion-pairing liquid chromatography using ultra-high performance LC (Thermo Scientific DionexUltiMate 3000) coupled with high-resolution/accurate-mass spectrometer (Thermo Scientific Q Exactive quadrupole-Orbitrap hybrid mass spectrometer) with electrospray ionization operated in negative mode. An injection sample of 10 μL was used and the column temperature was set to 25°C. A Waters Acquity UPLC BEH C_{18} 1.7 μm with a column size of 2.1 × 100 mm) (Waters Corporation, Massachusetts) was used. Solvent A contained 97:3 (v:v) LC-MS grade H_2O: methanol with acetic acid (15 mM) and tributylamine (10 mM). Solvent B contained 100% methanol. The flow rate was 180 μL min^{-1} during the entire sample run (25 min). The solvent gradient with respect to solvent A was the following: 0 min, 100%; 2.5 min, 100%; 5 min, 80%; 7.5 min, 80%; 10 min, 45%; 12 min, 45%; 14 min, 5%; 17 min, 5%; 18 min, 0%; 25 min, 0% was run. All metabolite identification and isotopic enrichment were determined using the Metabolomics Analysis and Visualization Engine (MAVEN) software package (Clasquin et al., 2012). Corrections for natural abundance of ^{13}C were conducted on the ^{13}C-labeled fractions.

Statistical Analysis

All experiments were conducted in three independent biological replicates. Unpaired two-tailed *t*-test analyses were conducted to evaluate statistically-significant differences between two

conditions. Statistical significance was determined for the following comparisons: specific growth rate of glyphosate-exposed cells compared to cells grown on succinate alone; changes in metabolite levels in *P. putida* S12 compared to levels in the other *Pseudomonas* species; labeled fraction of metabolites after 30-min of incorporated ^{13}C-labeled substrate in glyphosate-exposed KT2440 compared to control; and, specific growth rates of *P. putida* S12 cells grown on glyphosate-containing growth media with and without aromatic amino acids.

RESULTS

Growth Effects Are Both Strain- and Species-Dependent

Figure 2 illustrates the exponential growth rates determined from monitoring the growth of the four *Pseudomonas* species in medium containing succinate as the growth substrate in the absence and presence of glyphosate at 0.5 mM and 5 mM. For *P. putida* S12, the growth rate was reduced by 24.2 ± 2.7% ($p = 0.003$) during exposure to the low glyphosate dose compared to the control whereas growth was completely compromised with no cell growth obtained at the high glyphosate dose (**Figure 2A**). For *P. putida* KT2440, the growth rate at the low glyphosate dose was not statistically-significantly compared to the control (**Figure 2B**). However, at the high dose of glyphosate, the growth rate of the *P. putida* KT2440 cells was decreased by 23.4 ± 0.4% ($p = 0.02$) (**Figure 2B**). By contrast to the two *P. putida* strains, both *P. protegens* Pf-5 and *P. fluorescens* RA12 did not exhibit any adverse growth effects at both glyphosate exposures (**Figures 2C,D**). These data thus indicated that the *P. putida* species investigated here have higher sensitivity to glyphosate than the *P. fluorescens* and *P. protegens* species.

Relevant Glyphosate-Induced Widespread Metabolic Perturbations

To gain insights into the species-specific sensitivity to glyphosate, we profiled the metabolome of glyphosate-exposed cells and compared them to those obtained with cells-grown on succinate alone in the absence of glyphosate (**Figure 3**). We captured widespread perturbations in the metabolome of all species in response to glyphosate in the growth medium (**Figure 3**). Given the aforementioned highest sensitivity of *P. putida* S12 to glyphosate exposure, we used the metabolomic profiling of *P. putida* S12 as a reference to identify the possible metabolic perturbations responsible for the species-dependent inhibitory effects of glyphosate (**Figures 3**, **4**). We focused on the metabolites that were subjected to the most changes in *P. putida* S12, at least 10% different from the control in two or more of the three biological replicates in the presence of glyphosate (**Figures 3**, **4**). The relevant metabolites were associated with the shikimate pathway (shikimate-3-phosphate, tyrosine, tryptophan, phenylalanine), precursors to protein biosynthesis (tyrosine, tryptophan, phenylalanine, glutamine, valine), precursors of secondary metabolites (tryptophan, citrulline, ornithine), DNA biosynthesis (thymidine), and the tricarboxylic acid cycle (fumarate) (**Figures 3**, **4**).

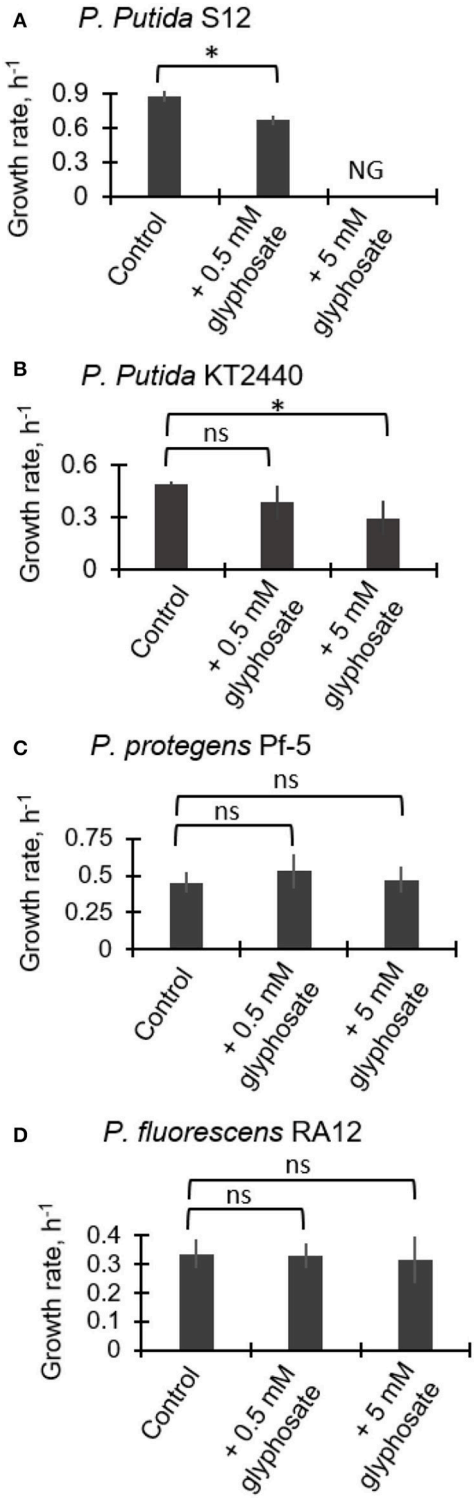


FIGURE 2 | Growth rates of succinate-grown *Pseudomonas* species in the absence (control) and presence of glyphosate (0.5 mM and 5 mM): **(A)** *P. putida* S12; **(B)** *P. putida* KT2440; **(C)** *P. protegens* Pf-5; **(D)** *P. fluorescens* RA12. Abbreviation for **(B)**: NG, no growth. The measured data were from three biological replicates ($n = 3$). Two-tailed unpaired *t*-test analysis comparing the specific growth rate of the control experiment to the growth rates of glyphosate-exposed cells: $p < 0.05$ (*); ns, not statistically significant.

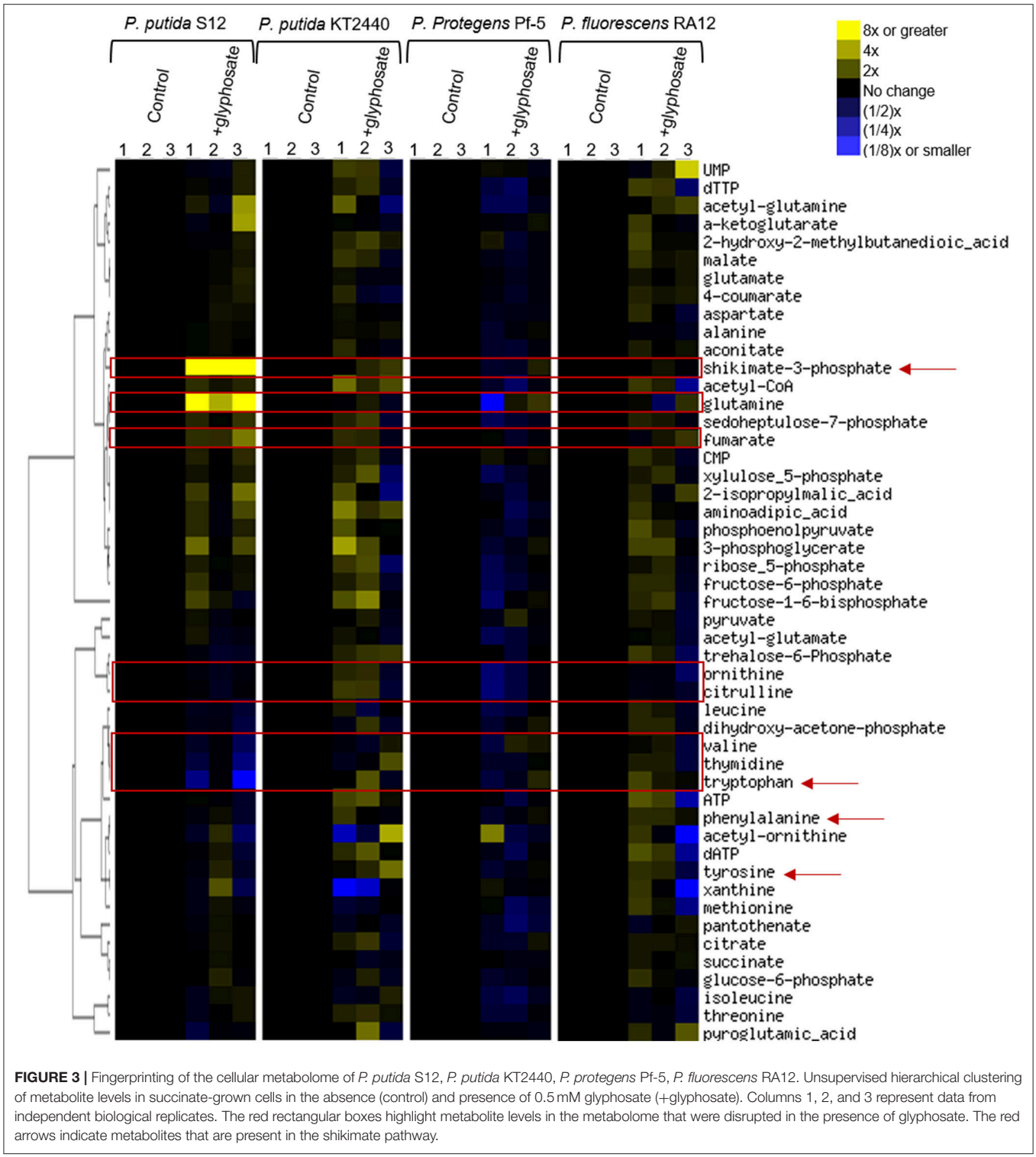


FIGURE 3 | Fingerprinting of the cellular metabolome of *P. putida* S12, *P. putida* KT2440, *P. protegens* Pf-5, *P. fluorescens* RA12. Unsupervised hierarchical clustering of metabolite levels in succinate-grown cells in the absence (control) and presence of 0.5 mM glyphosate (+glyphosate). Columns 1, 2, and 3 represent data from independent biological replicates. The red rectangular boxes highlight metabolite levels in the metabolome that were disrupted in the presence of glyphosate. The red arrows indicate metabolites that are present in the shikimate pathway.

In glyphosate-exposed *P. putida* S12, we observed depletion in the levels of phenylalanine, ornithine, citrulline, valine, thymidine, and tryptophan (**Figure 3**). Relative to *P. putida* S12 cells grown on succinate alone, the levels of phenylalanine, ornithine, and citrulline were only about 10 to 15% less in cells grown on the glyphosate-containing growth medium (**Figure 4**). There was a more significant depletion of the other metabolites in *P. putida* S12 in response to glyphosate exposure: valine and thymidine were 35 to 60% less relative to the control and there was an about 5-fold reduction in the tryptophan pool relative

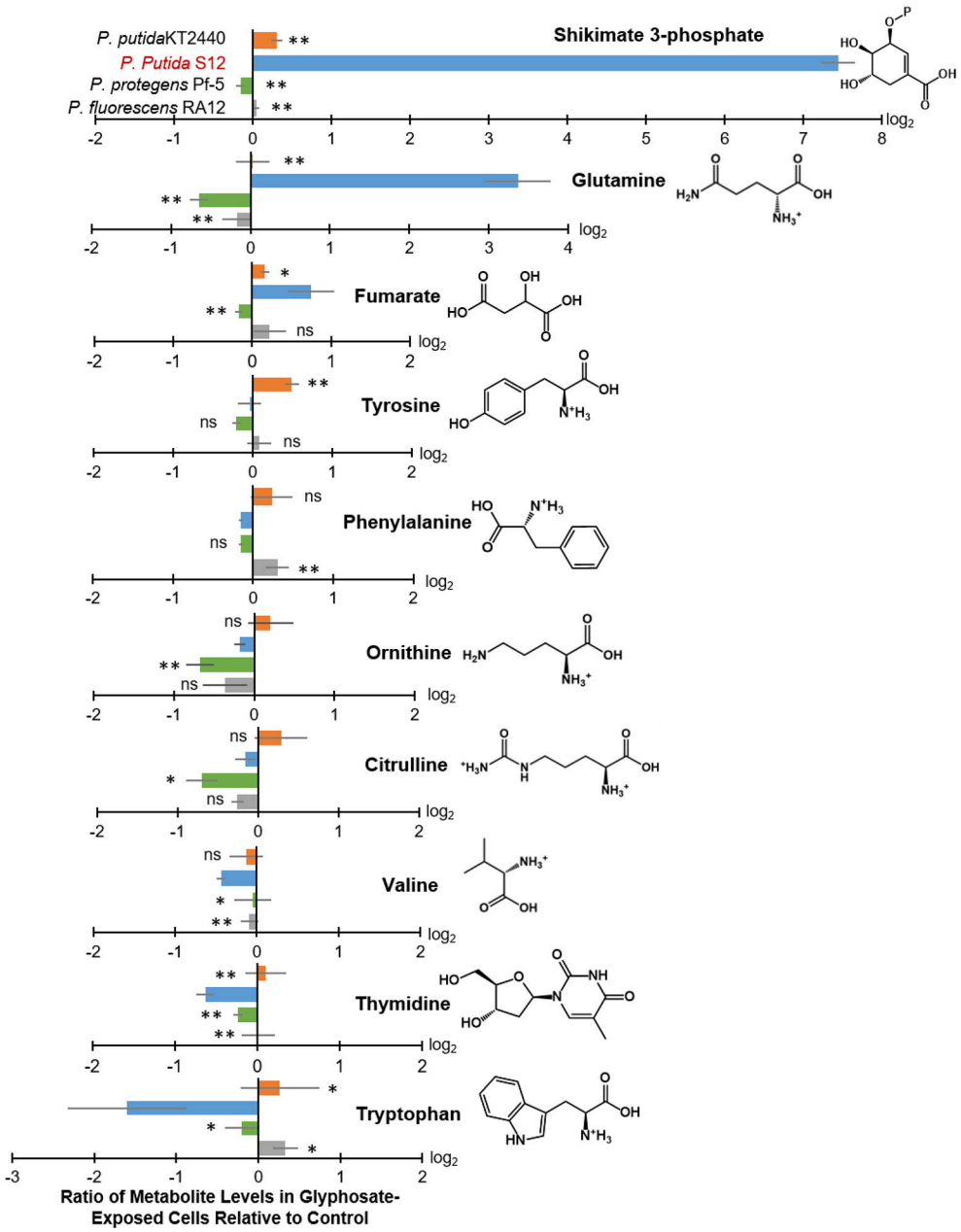


FIGURE 4 | Intracellular levels of selected metabolites in glyphosate-exposed cells relative to control. Extracted from the cellular metabolome shown in **Figure 3** are $\log_2$-tranformed ratios of metabolite levels in glyphosate-exposed cells (at 0.5 mM) relative to control with statistically-significant differences across the different *Pseudomonas* species: *P. putida* KT2440 (orange), *P. putida* S12 (light blue), *P. protegens* Pf-5 (green), and *P. fluorescens* RA12 (gray). Data (average ± standard deviation) were obtained from three independent biological replicates. Two-tailed unpaired *t*-test analysis comparing the ratios obtained with *P. putida* S12 (the most sensitive species) to those obtained with each of the other species: $p < 0.01$ (**), $p < 0.05$ (*), not statistically significant (ns).

to the control (**Figure 4**). In regards to the other species, there was no statistical difference between the changes in the levels of both ornithine and citrulline recorded in glyphosate-exposed *P. putida* S12 and the corresponding changes in *P. putida* KT2440, *P. protegens* Pf-5, and *P. fluorescens* RA12 (**Figure 4**); the change in phenylalanine was either the same as the one recorded for *P. putida* S12 (*P. putida* KT2440 and *P. protegens* Pf-5) or slightly elevated (up to 40% more in *P. fluorescens* RA12) (**Figure 4**). Most notably, the changes in the levels of valine, thymidine and tryptophan in the other *Pseudomonas* species in response to glyphosate were different from the significant depletion obtained with *P. putida* S12 ($p < 0.05$, $n = 3$): valine in the presence of glyphosate remained the same as in the control condition in the other species; there was either no change in thymidine or

a small decrease in the presence of glyphosate; and, tryptophan remained the same or was slightly elevated in the other species (**Figure 4**). These data implied that the severe depletion in these three metabolites (valine, thymidine, and tryptophan) specifically in *P. putida* S12 may be important in mediating the higher glyphosate toxicity in this bacterium.

In addition to the decrease in the levels of selected metabolites, we obtained a significant buildup of the following metabolites in glyphosate-exposed *P. putida* S12: shikimate-3-phosphate (up to nearly 200-fold increase), glutamine (up to nearly 16-fold increase), and fumarate (up to 2-fold increase) (**Figure 4**). The levels of both shikimate-3-phosphate and fumarate were also found to be elevated in *P. putida* KT2440 (**Figure 4**). Specifically, shikimate-3-phosphate was about 23% higher and fumarate was about 10% higher than the control in *P. putida* KT2440 (**Figure 4**). In contrast, *P.* protegens Pf-5 and *P. fluorescens* RA12 grown in the presence of glyphosate did not present any change in the shikimate-3-phosphate level and exhibited no change to a slight increase in the fumarate level (**Figure 4**). Interestingly, the change in the aromatic AAs was not the same in *P. putida* S12 (**Figure 4**). While there was a severe depletion in tryptophan (up to 5-fold decrease) and a modest depletion in phenylalanine (up to 12% decrease), the level of tyrosine remained unchanged in *P. putida* S12 in response to glyphosate (**Figure 4**). Whereas, the change in the tyrosine level in both *P. protegens* Pf-5 and *P. putida* KT2440 was not statistically different from *P. putida* S12, *P. putida* KT2440 had up to 43% increase in tyrosine level in response to glyphosate exposure.

These metabolomics data implied that, in addition to the shikimate pathway, widespread perturbations in the metabolome may exacerbate effects of glyphosate (**Figures 3**, **4**). However, the highest accumulation of shimate-3-phosphate and the greatest depletion of tryptophan pointed specifically to the shikimate pathway as the potential primary toxicity target, as has been proposed previously in other *Pseudomonas* species (Schulz et al., 1985).

Glyphosate Specifically Targets Aromatic Amino Acid Biosynthesis

It was previously reported that the inhibitory effect of glyphosate on EPSP synthase activity in different *Pseudomonas* species was species-dependent (Schulz et al., 1985). As a consequence of the inhibition of EPSP synthase in the glyphosate-exposed cells, disruption in downstream metabolic fluxes in the shikimate pathway was expected, specifically in regards to the biosynthesis of the three aromatic AAs (phenylalanine, tyrosine, and tryptophan) downstream this pathway. Focusing on *P. protegens* Pf-5 and *P. putida* KT2440, we employed a kinetic flux profiling approach using [U-$^{13}C_4$]-succinate to evaluate explicitly the biosynthesis of aromatic AA as a specific toxicological target of glyphosate (**Figure 5**). In this approach, evaluation of *in vivo* flux through a metabolic reaction in the pathway was considered on the basis that the flux would be proportional to the level of the reactant metabolite and the labeling kinetics of the product metabolite (Yuan et al., 2008; Sasnow et al., 2016). Kinetic incorporation of the labeled succinate in both species was monitored at both the low-dose and high-dose glyphosate exposures and we found that the labeling kinetics remained the same in the absence and presence of glyphosate (**Figure 5**). These results indicated that the uptake of the organic carbon source (i.e., succinate) by the cells was not compromised by the presence of glyphosate. We also found that the labeling kinetics of both PEP, the endogenous analog of glyphosate, and shikimate-3-phosphate remained the same in the glyphosate-exposed cells (**Figure 5**), thus indicating that the synthesis of these metabolites was not affected by glyphosate exposure.

On the other hand, we obtained both species-dependent and dose-dependent effects of glyphosate on the biosynthetic fluxes of the aromatic AAs (**Figure 5**). At the range of glyphosate concentrations investigated here, we did not record any change in the biosynthetic fluxes of all three aromatic AAs in *P. protegens* Pf-5 (**Figure 5A**). However, for *P. putida* KT2440, the ^{13}C isotopic flux in the glyphosate-exposed cells clearly demonstrated an effect of glyphosate exposure compared to the control experiment (**Figure 5B**). For phenylalanine, the labeling kinetics remained unchanged following exposure of *P. putida* KT2440 to the low glyphosate dose (**Figure 5B**). But, when the *P. putida* KT2440 cells were grown in medium with the high glyphosate dose, the labeling kinetics of phenylalanine remained unchanged initially but there was an approximate 22% decrease in the total fraction of fully labeled phenylalanine (**Figure 5B**). Compared to *P. putida* KT2440 cells grown on succinate alone, a slower labeling kinetics was observed in the biosynthetic flux of both tyrosine and tryptophan in the presence of glyphosate (**Figure 5B**). By 30 min, the total fraction of fully labeled tyrosine was decreased by 21 and 46% at the low-dose and high-dose glyphosate exposures, respectively (**Figure 5B**). The corresponding decrease for labeled tryptophan was 43 and 52%, respectively (**Figure 5B**). In accordance with our data, the increasing order of impaired biosynthetic flux with respect to the shikimate-associated aromatic AAs in *P. putida* KT 2440 in the presence of glyphosate was phenylalanine, tyrosine, and tryptophan (**Figure 5B**).

Exogenous Supply of Aromatic Amino Acids Overcomes Lethal Glyphosate Dose

Our metabolomics data revealed widespread changes in metabolite levels from different pathways in the cellular metabolome (**Figure 4**). And, kinetic ^{13}C isotopic flux data with glyphosate-exposed cells demonstrated specific disruption of metabolic fluxes through the shikimate pathway, thereby confirming that the well-known toxicity of glyphosate in plants via the inhibition of the biosynthesis of aromatic AAs was also evident in *P. putida* KT2440, even at sub-lethal glyphosate exposure (**Figure 5B**). As previously discussed, amongst the *Pseudomonas* species investigated here, only the *P. putida* S12 cells exhibited complete growth inhibition in response to glyphosate exposure, specifically in the high-glyphosate condition (**Figure 2A**). To determine whether the lethal consequence of glyphosate (at 5 mM) in *P. putida* S12 was due primarily to a deficiency in the biosynthetic supply of aromatic AAs, we repeated the growth experiments with a growth mixture

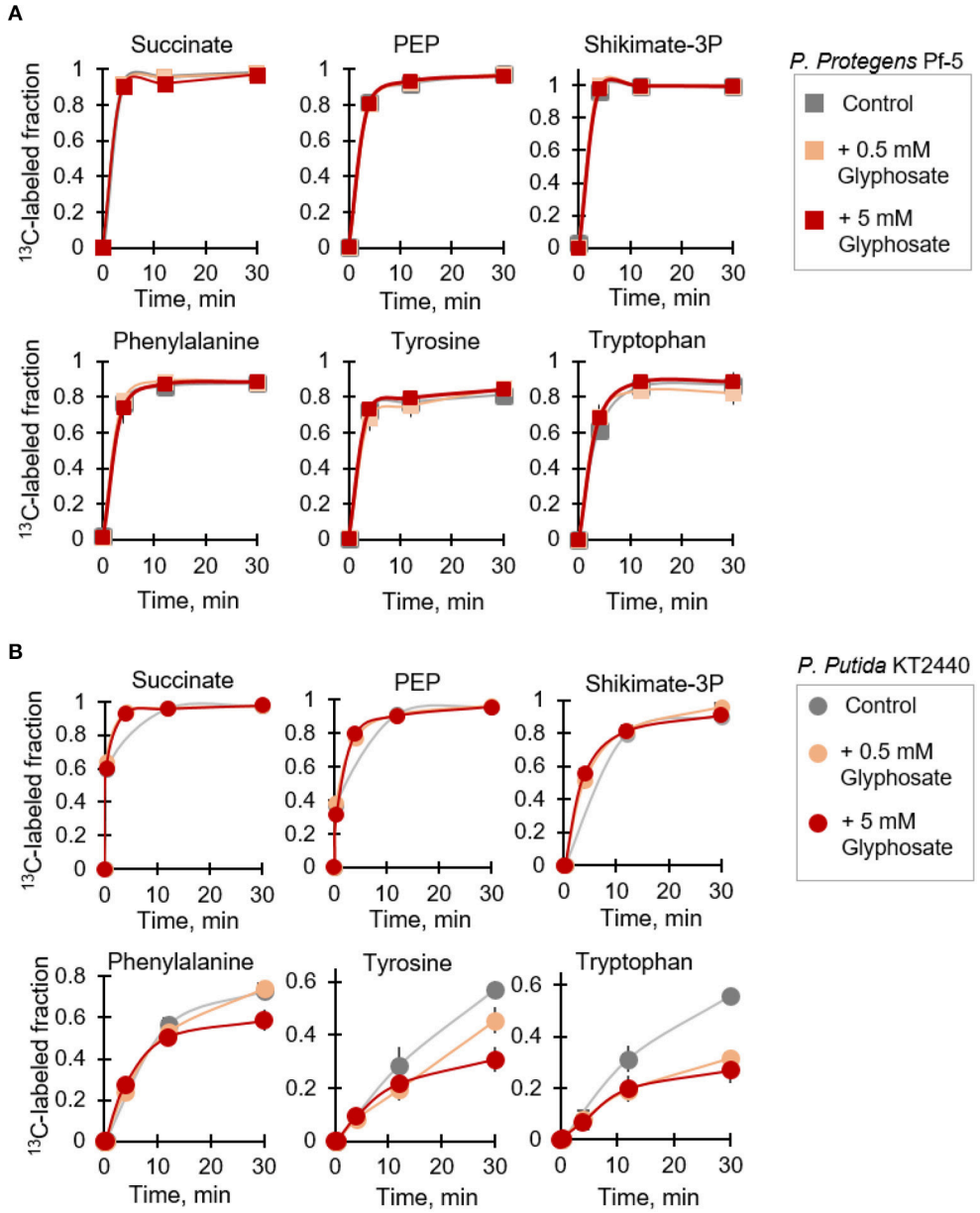


FIGURE 5 | Kinetic isotopic profiling of the shikimate pathway of glyphosate-exposed *Pseudomonas* species. Dynamic biosynthesis of fully-labeled fractions of metabolites in *P. protegens* Pf-5 **(A)** and *P. putida* KT2440 **(B)** following isotopic switch from unlabeled succinate to ^{13}C-labeled substrate in the absence (control) or in the presence of glyphosate (at 0.5 mM or 5 mM). The data (average ± standard deviation) are from independent biological replicates ($n = 3$). Error bars are not visible when they are very small. Compared to the labeling kinetics in the control experiment, the labeling fraction of the aromatic amino acids at 30-min in the *P. putida* KT2440 cells at the high-glyphosate concentration was statistically-significant ($p < 0.05$).

that contains all three aromatic AAs (tryptophan, tyrosine, phenylalanine) (**Figure 6A**). Remarkably, the exogenous supply of aromatic AAs in the growth medium led to the complete recovery of the cell growth (**Figure 6A**). Specifically, the recovered growth rate at $0.61 \pm 0.17\,h^{-1}$ in the glyphosate-containing growth medium supplemented with the aromatic AAs was similar, within an error imprecision of one standard deviation, to the growth rate ($0.88 \pm 0.11\,h^{-1}$) of the cells in the absence of glyphosate (**Figure 6A**). These results implied that *P. putida* S12 cells lost the biosynthetic ability to produce aromatic AAs in the presence of high glyphosate dose and thus must rely on extracellular supply to survive.

In addition, we also probe whether the exogenous supply aromatic AAs also led to the recovery of metabolic disruptions (**Figure 6B**). The buildup in shikimate-3-phosphate that was captured in the glyphosate-exposed cells in the absence of the aromatic AAs remained in the cells after the supplement of aromatic AA (**Figure 4**). This was consistent with continued

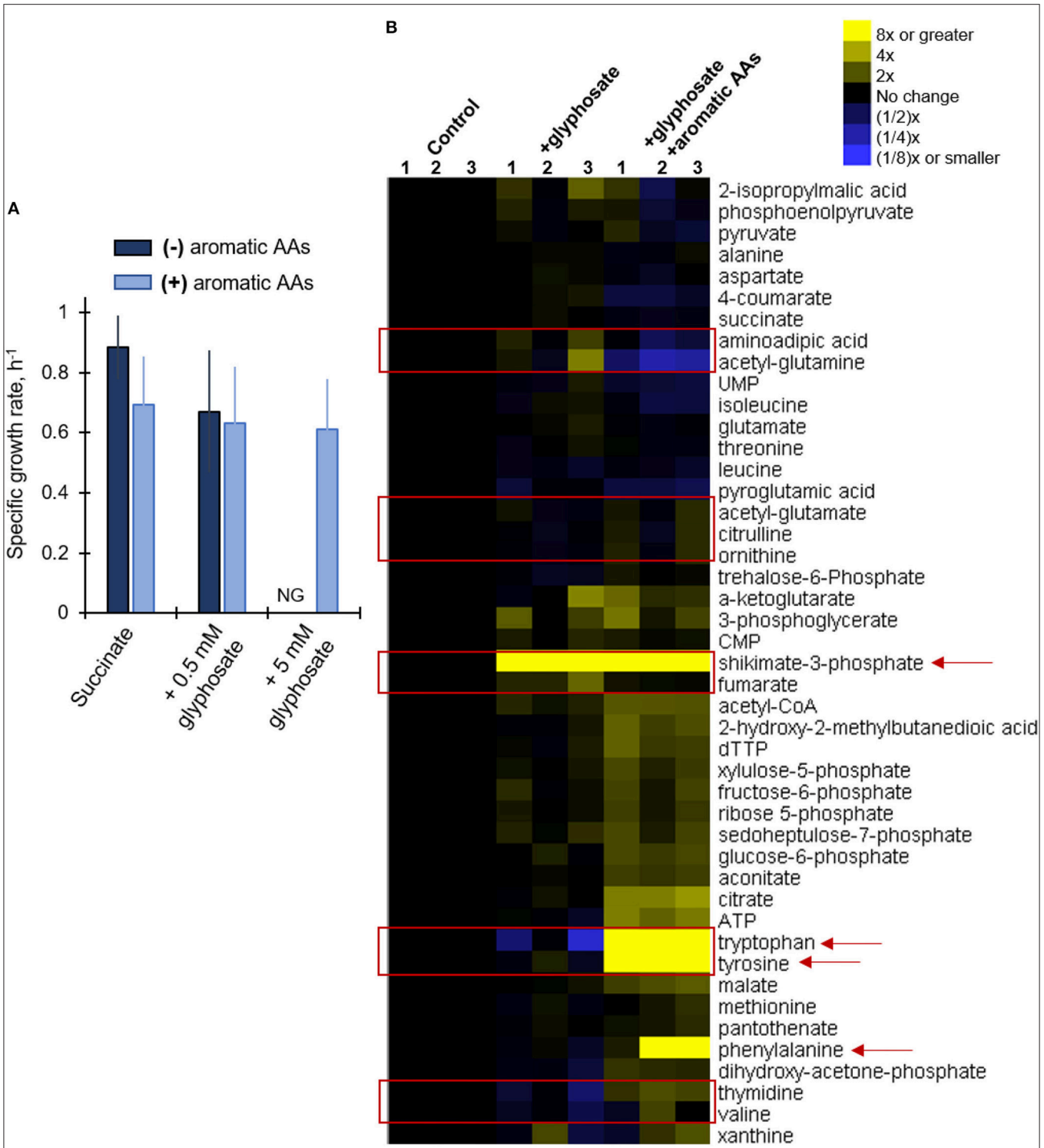


FIGURE 6 | Growth and metabolic phenotypes of glyphosate-exposed *P. putida* S12 to supplementation of aromatic amino acids in the growth media. **(A)** Growth rates (average ± standard deviation) of succinate-grown and glyphosate-exposed cells without (dark blue) and with (light blue) added aromatic amino acids (AAs) in the growth medium); see materials and methods for more details. **(B)** Unsupervised hierarchical clustering of metabolite levels in succinate-grown cells in the absence (control), presence of 0.5 mM glyphosate (+glyphosate) or the lethal glyphosate dose, 5 mM, and aromatic AAs (+glyphosate +aromatic AAs). Columns 1, 2, and 3 represent data from independent biological replicates. The red rectangular boxes highlight metabolite levels in the metabolome that were disrupted in the presence of glyphosate. The red arrows indicate metabolites that are present in the shikimate pathway. Abbreviation for **(A)**: NG, no growth. The measured data in **(A)** were from three biological replicates ($n = 3$).

inhibition of the EPSP synthase by glyphosate (**Figure 1B**). However, the downstream effect of this inhibition was circumvented by the accumulation of all three aromatic AAs in the cellular metabolome due to the exogenous supply of these AAs in the growth medium (**Figure 6B**). Our metabolomics profiling of *P. putida* S12 at the lethal glyphosate level (5 mM) with aromatic AAs revealed that the supplement of aromatic AAs restored the levels of several metabolites beyond those associated with the shikimate pathway (**Figure 6B**). Specifically, the levels of both the nucleoside thymidine and the AA valine, which were shown to be depleted in *P. putida* S12 in the presence of glyphosate, restored either to the same level as the control or were slightly elevated in response to the aromatic AA supplement (**Figure 6B**). We also found that the level of fumarate was restored to the level of the control in cells grown on media containing both glyphosate and the aromatic AAs (**Figure 6B**). Other major changes in the metabolome of *P. putida* S12 were also observed when comparing the glyphosate-exposed cells grown in the absence and in the presence of the aromatic AA supplement (**Figure 6**). Notably, the levels of citrulline, ornithine, acetyl-glutamate increased in the presence of excess aromatic AAs (**Figure 6B**). On the other hand, the levels of aminoadipic acid and acetyl-glutamine decreased substantially in the presence of excess aromatic AAs (**Figure 6B**). We are not able to determine the exact relevance of these changes but they suggest that the cellular level of aromatic AAs was connected to the metabolic balance of other amino acids or amino acid-containing metabolites.

DISCUSSION

As stated in the Introduction, we postulated three hypotheses underlying the sensitivity of soil *Pseudomonas* species to glyphosate exposure: (1) species-specific growth inhibitory effects, (2) inhibition of the shikimate pathway, and (3) disruption of other cellular pathways. In relation to the first hypothesis, we show here that within the same *Pseudomonas* genus, the growth responses of glyphosate-exposed cells were both species-dependent and strain-dependent (**Figure 2**). Notably, the growth of both the *P. protegens* and *P. fluorescens* species used here was not affected by the range of glyphosate concentration added to the growth medium (**Figure 2**). By contrast, the *P. putida* species were more sensitive to the glyphosate-containing growth media whereby, compared to growth in the absence of glyphosate, the strain S12 experienced up to 100% growth inhibition but the strain KT2440 only exhibited up to about 23% growth inhibition (**Figure 2**). There have been conflicting results on the adverse effects of glyphosate exposure on soil microbial community structure (Weaver et al., 2007; Johal and Huber, 2009; Kremer and Means, 2009; Mijangos et al., 2009; Barriuso et al., 2011; Duke et al., 2012). Our results here support the proposal that species-dependent sensitivities to glyphosate led to the observed change in microbial community structure in soils amended with glyphosate (Ratcliff et al., 2006; Newman et al., 2016). It was found that six different genera of cyanobacteria responded differently to glyphosate concentrations (from 0.03 to 10 mM) and the growth response ranged from no change to lethal effects (Forlani et al., 2008). Along with our findings, these previous reports implied that the wide range of sensitivities of different genera and species of microorganisms will lead to dissimilar effects of glyphosate on different ecosystems.

We evaluated the second and third hypotheses by profiling the cellular metabolome, which revealed species-dependent widespread metabolic disruptions (**Figure 3**). An accumulation in the levels of metabolites upstream of the EPSP synthase was captured in time-course experiments of glyphosate-exposed pea plant *Pisum sativum* (Zabalza et al., 2016). We note that the metabolite shikimate-3P is upstream of EPSP synthase, the targeted enzyme in glyphosate-induced toxicity in plants (**Figure 1B**). Therefore, the buildup of shikimate-3P in *P. putida* KT2440 and *P. putida* S12 was indicative of inhibition of EPSP synthase activity by glyphosate in these species (Schönbrunn et al., 2001) (**Figures 3**, **4**). Interestingly, hierarchical clustering of the metabolome indicated that the disruption in non-shikimate pathway metabolites was clustered with several of the shikimate pathway-associated metabolites (**Figure 3**). Specifically, elevated glutamine and fumarate levels in *P. putida* S12 were clustered with the significant accumulation of shikimate-3-phosphate (**Figure 3**). And, the significant depletion in tryptophan level was clustered with depletion of valine and thymidine (**Figure 3**). In accordance with our results, impairment of pathways involved in energy metabolism (Orcaray et al., 2012) and protein synthesis (Maroli et al., 2016) were found in glyphosate-treated plants. Intracellular accumulation of AAs and depletion in protein synthesis have been reported for the glyphosate-sensitive biotype of the flowering plant *Amaranthus palmeri* (Maroli et al., 2016). This was proposed to be due to the metabolic response of the plant to the abiotic stressor (Maroli et al., 2016), which may include a decrease in *de novo* protein synthesis. Thus, we attributed the significant accumulation of glutamine in the glyphosate-sensitive *P. putida* S12 to either inhibition of protein synthesis or disruption of other glutamine-consuming metabolic pathways by glyphosate (**Figure 4**). By contrast, in *P. protegens* Pf-5 and *P. fluorescens* RA12, there was a depletion in glutamine in the glyphosate-exposed cells, thus indicating that glutamine-consuming pathways were less inhibited in these cells (**Figure 4**). Furthermore, our results with the two species most sensitive to glyphosate (*P. putida* S12 and *P. putida* KT2440) indicated a more severe effect on the level of tryptophan than the other two aromatic AAs (**Figure 4**). This disproportionate effect on the pools of aromatic AAs in *P. putida* KT2440 may be due to the differential role of each amino acid in the cellular response to glyphosate exposure. Further metabolic studies are needed to verify this proposal.

Our ^{13}C kinetic flux profiling data revealed a species-dependent inhibition of biosynthetic fluxes of the aromatic AAs that are synthesized downstream of the shikimate pathway (**Figure 5**). Whereas, these fluxes remained the same in *P. protegens* Pf-5 upon exposure to glyphosate, we obtained up to 60% decrease in the final isotopic incorporation in the aromatic AAs in *P. putida* KT2440 (**Figure 5**). This species-dependent difference in the ^{13}C kinetic flux was consistent with the species-dependent growth responses to glyphosate (**Figures 2**, **5**).

Supplementation of growth medium with aromatic AAs led to complete growth recovery of *P. putida* S12 exposed to lethal dose of glyphosate (**Figure 6A**). These results implied that the specific impairment of *de novo* biosynthesis of aromatic AAs was primarily responsible for the growth effects on the different *Pseudomonas* species. And subsequent metabolomics profiling demonstrated that the aromatic AA supplementation also led to the recovery of metabolic homeostasis as well as changes that deviated from the control condition (**Figure 6B**). Our findings suggest that rhizospheric bacteria may be less susceptible to glyphosate effects due to the high-carbon environment in the rhizosphere with relatively higher concentration of amino acids and sugars relative to bulk soils.

Four important caveats should be considered in assessing the relevance of our findings. First, we concluded from our data that the inhibition of the biosynthesis of aromatic AAs was a specific target of glyphosate. The different glyphosate-induced growth effects and metabolic phenotypes implied different sensitivities of the metabolome, in addition to the shikimate pathway. In fact, it was found previously that the inhibition of the activity of EPSP synthase, an important enzyme in the initiation of the shikimate pathway, was greater in *P. fluorescens* AFT36 than in *P. putida* W1616 (Schulz et al., 1985). We also note that additional factors may be contributing to these differences such as different cell permeability to cellular glyphosate uptake and different ability to break down glyphosate (Kishore and Jacob, 1987; Liu et al., 1991; Zboińska et al., 1992). Second, *Pseudomonas* species are known to produce auxin-like plant hormones including IAA and antifungal metabolites such as pyrrolnitrin, both of which rely on the aromatic AA tryptophan as an important metabolite precursor derived from the shikimate pathway. It remains to be determined whether the biosynthetic pathway of these plant growth-beneficial molecules is negatively affected by glyphosate treatment due to depletion of aromatic AAs. Third, our study focused on glyphosate, the parental active herbicide ingredient. It is important to note that aminomethylphosphonic acid, the primary degradation product of glyphosate, has been widely detected along with glyphosate in environmental matrices (Reddy et al., 2008; Battaglin et al., 2014). Moreover, glyphosate-based herbicide formulations contain a number of additives including surfactants. An evaluation of synergistic or counteracting effects of glyphosate, its breakdown products, and surfactants is warranted. Fourth, several soil *Pseudomonas* species are beneficial players in the plant microbiome near the rhizosphere. In addition to these species, we recognize that a diverse number of bacterial and fungal genera exist in this microbiome. A comprehensive understanding of the responses of the entire rhizospheric microbiome is required to assess fully the non-targeted metabolic effects of glyphosate on crop-beneficial microbial communities.

AUTHOR CONTRIBUTIONS

LA supervised the research, conducted extensive analysis of data, and wrote the manuscript. LA, MLR, RAW, and MAK designed the research. MLR, VK, RAW, and CRSS conducted the different growth experiments. MLR, RAW, and TY conducted the metabolomics experiments. All authors contributed to preliminary data analysis and provided edits to drafts of the manuscript.

ACKNOWLEDGMENTS

Graduate support for RAW, TY, and MAK was provided, respectively, by a graduate fellowship from Cornell University, a scholarship from the Schooner foundation, and an Integrative Graduate Education and Research Traineeship (IGERT) research fellowship from the National Science Foundation. Undergraduate support for MLR, VK, and CRSS was provided by the National Institute of Food and Agriculture (Hatch project 1237419) and an Academic Venture Fund from the Cornell Atkinson Center for a Sustainable Future. This research was supported also in part by a start-up package from Cornell University.

REFERENCES

Aristilde, L. (2017). Metabolite labeling reveal hierarchies in Clostridium acetobutylicum that selectively channels carbons from sugar mixtures towards biofuel precursors. *Microb. Biotechnol.* 10, 162–174. doi: 10.1111/1751-7915.12459

Barriuso, J., Marín, S., and Mellado, R. P. (2011). Potential accumulative effect of the herbicide glyphosate on glyphosate-tolerant maize rhizobacterial communities over a three-year cultivation period. *PLoS ONE* 6:e27558. doi: 10.1371/journal.pone.0027558

Battaglin, W. A., Meyer, M. T., Kuivila, K. M., and Dietze, J. E. (2014). Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. *J. Am. Water Resour. Assoc.* 50, 275–290. doi: 10.1111/jawr.12159

Clasquin, M., Melamud, E., and Rabinowitz, J. D. (2012). LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. *Curr. Prot. Bioinform*. 37, 14111–141123.

Druille, M., Cabello, M. N., García Parisi, P. A., Golluscio, R. A., and Omacini, M. (2015). Glyphosate vulnerability explains changes in root-symbionts propagules viability in pampean grasslands. *Agric. Ecosyst. Environ.* 202, 48–55. doi: 10.1016/j.agee.2014.12.017

Druille, M., Cabello, M. N., Omacini, M., and Golluscio, R. A. (2013). Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi. *Appl. Soil Ecol.* 64, 99–103. doi: 10.1016/j.apsoil.2012.10.007

Duke, S. O., Lydon, J., Koskinen, W. C., Moorman, T. B., Chaney, R. L., and Hammerschmidt, R. (2012). Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. *J. Agric. Food Chem.* 60, 10375–10397. doi: 10.1021/jf302436u

Forlani, G., Pavan, M., Gramek, M., Kafarski, P., and Lipok, J. (2008). Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. *Plant Cell. Physiol.* 49, 443–456. doi: 10.1093/pcp/pcn021

Funke, T., Han, H., Healy-Fried, M. L., Fischer, M., and Schönbrunn, E. (2006). Molecular basis for the herbicide resistance of roundup ready crops. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13010–13015. doi: 10.1073/pnas.0603638103

Johal, G. S., and Huber, D. M. (2009). Glyphosate effects on diseases of plants. *Eur. J. Agron.* 31, 144–152. doi: 10.1016/j.eja.2009.04.004

Johal, G. S., and Rahe, J. E. (1984). Effect of soilborne plant-pathogenic fungi on the herbicidal action of glyphosate on bean seedlings. *Phytopathology* 74, 950–955. doi: 10.1094/Phyto-74-950

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J., et al. (1998). Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. *J. Bacteriol.* 180, 1939–1943.

Kishore, G. M., and Jacob, G. S. (1987). Degradation of glyphosate by *Pseudomonas* sp. PG2982 via a sarcosine intermediate. *J. Biol. Chem.* 262, 12164–12168.

Kremer, R. J., and Means, N. E. (2009). Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms. *Eur. J. Agron.* 31, 153–161. doi: 10.1016/j.eja.2009.06.004

Kremer, R., Means, N., and Kim, S. (2005). Glyphosate affects soybean root exudation and rhizosphere micro-organisms. *Int. J. Environ. Anal. Chem.* 85, 1165–1174. doi: 10.1080/03067310500273146

Lancaster, S. H., Hollister, E. B., Senseman, S. A., and Gentry, T. J. (2010). Effects of repeated glyphosate applications on soil microbial community composition and the mineralization of glyphosate. *Pest. Manag. Sci.* 66, 59–64. doi: 10.1002/ps.1831

Lane, M., Lorenz, N., Saxena, J., Ramsier, C., and Dick, R. P. (2012). The effect of glyphosate on soil microbial activity, microbial community structure, and soil potassium. *Pedobiologia* 55, 335–342. doi: 10.1016/j.pedobi.2012.08.001

Larson, R. L., Hill, A. L., Fenwick, A., Kniss, A. R., Hanson, L. E., and Miller, S. D. (2006). Influence of glyphosate on rhizoctonia and fusarium root rot in sugar beet. *Pest Manag. Sci.* 62, 1182–1192. doi: 10.1002/ps.1297

Lévesque, C. A., Rahe, J. E., and Eaves, D. M. (1987). Effects of glyphosate on *Fusarium* spp.: its influence on root colonization of weeds, propagule density in the soil, and crop emergence. *Can. J. Microbiol.* 33, 354–360. doi: 10.1139/m87-062

Lévesque, C. A., and Rahe, J. R. (1992). Herbicide Interactions with Fungal root pathogens, with special reference to glyphosate. *Annu. Rev. Phytopathol.* 30, 579–602. doi: 10.1146/annurev.py.30.090192.003051

Liu, C.-M., McLean, P. A., Sookdeo, C. C., and Cannon, F. C. (1991). Degradation of the herbicide glyphosate by members of the family rhizobiaceae. *Appl. Environ. Microbiol.* 57, 1799–1804.

Maroli, A., Nandula, V., Duke, S., and Tharayil, N. (2016). Stable isotope resolved metabolomics reveals the role of anabolic and catabolic processes of glyphosate-induced amino acid accumulation in *Amaranthus palmeri* biotypes. *J. Agric. Food Chem.* 64, 7040–7048. doi: 10.1021/acs.jafc.6b02196

Mijangos, I., Becerril, J. M., Albizu, I., Epelde, L., and Garbisu, C. (2009). Effects of glyphosate on rhizosphere soil microbial communities under two different plant compositions by cultivation-dependent and -independent methodologies. *Soil Biol. Biochem.* 41, 505–513. doi: 10.1016/j.soilbio.2008.12.009

Newman, M. M., Hoilett, N., Lorenz, N., Dick, R. P., Liles, M. R., Ramsier, C., et al. (2016). Glyphosate effects on soil rhizosphere-associated bacterial communities. *Sci. Tot. Environ.* 543, 155–160. doi: 10.1016/j.scitotenv.2015.11.008

Orcaray, L., Zulet, A., Zabalza, A., and Royuela, M. (2012). Impairment of carbon metabolism induced by the herbicide glyphosate. *J. Plant Physiol.* 169, 27–33. doi: 10.1016/j.jplph.2011.08.009

Ratcliff, A. W., Busse, M. D., and Shestak, C. J. (2006). Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. *Appl. Soil. Ecol.* 34, 114–124. doi: 10.1016/j.apsoil.2006.03.002

Reddy, K. N., Rimando, A. M., Duke, S. O., and Nandula, V. K. (2008). Aminomethylphosphonic acid accumulation in plant species treated with glyphosate. *J. Agric. Food Chem.* 56, 2125–2130. doi: 10.1021/jf072954f

Santos, A., and Flores, M. (1995). Effects of glyphosate on nitrogen fixation of free-living heterotrophic bacteria. *Lett. Appl. Microbiol.* 20, 349–352. doi: 10.1111/j.1472-765X.1995.tb01318.x

Sasnow, S. S., Wei, H., and Aristilde, L. (2016). Bypasses in intracellular glucose metabolism in iron-limited *Pseudomonas putida*. *Microbiologyopen* 5, 3–20. doi: 10.1002/mbo3.287

Schönbrunn, E., Eschenburg, S., Shuttleworth, W. A., Schloss, J. V., Amrhein, N., Evans, J. N., et al. (2001). Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1376–1380. doi: 10.1073/pnas.98.4.1376

Schulz, A., Krüper, A., and Amrhein, N. (1985). Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. *FEMS Microbiol. Lett.* 28, 297–301. doi: 10.1111/j.1574-6968.1985.tb00809.x

Shehata, A. A., Schrödl, W., Aldin, A. A., Hafez, H. M., and Krüger, M. (2013). The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota *in vitro*. *Curr. Microbiol.* 66, 350–358. doi: 10.1007/s00284-012-0277-2

Steinrücken, H. C., and Amrhein, N. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochem. Biophys. Res. Commun.* 94, 1207–1212. doi: 10.1016/0006-291X(80)90547-1

Timmis, K. N. (2002). *Pseudomonas putida*: a cosmopolitan opportunist par excellence. *Environ. Microbiol.* 4, 779–781. doi: 10.1046/j.1462-2920.2002.00365.x

Tzin, V., and Galili, G. (2010). New insights into the shikimate and aromatic amino acids biosynthesis pathways. *Mol. Plant* 3, 956–972. doi: 10.1093/mp/ssq048

Weaver, M. A., Krutz, L. J., Zablotowicz, R. M., and Reddy, K. N. (2007). Effects of glyphosate on soil microbial communities and its mineralization in a Mississippi soil. *Pest Manag. Sci.* 63, 388–393. doi: 10.1002/ps.1351

Woodburn, A. T. (2000). Glyphosate: production, pricing and use worldwide. *Pest Manag. Sci.* 56, 309–312. doi: 10.1002/(SICI)1526-4998(200004)56:4<309::AID-PS143>3.0.CO;2-C

Yuan, J., Bennett, B. D. and Rabinowitz, J. D. (2008). Kinetic flux profiling for quantitation of cellular metabolic fluxes. *Nat. Protoc.* 3, 1328–1340. doi: 10.1038/nprot.2008.131

Zabalza, A., Orcaray, L., Fernández-Escalada, M., Zulet-González, A., and Royuela, M. (2016). The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots. *Pest. Biochem. Phys.* doi: 10.1016/j.pestbp.2016.12.005. [Epub ahead of print].

Zboińska, E., Lejczak, B., and Kafarski, P. (1992). Organophosphonate utilization by the wild-type strain of *Pseudomonas fluorescens*. *Appl. Environ. Microbiol.* 58, 2993–2999.

Zhao, Y. (2012). Auxin biosynthesis: a simple two-step pathway converts tryptophan to indole-3-acetic acid in plants. *Mol. Plant* 5, 334–338. doi: 10.1093/mp/ssr104

Zobiole, L. H., Kremer, R. J., Oliveira, R. S. Jr., and Constantin, J. (2011). Glyphosate affects micro-organisms in rhizospheres of glyphosate-resistant soybeans. *J. Appl. Microbiol.* 110, 118–127. doi: 10.1111/j.1365-2672.2010.04864.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Aristilde, Reed, Wilkes, Youngster, Kukurugya, Katz and Sasaki. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 14 February 2018
doi: 10.3389/fenvs.2018.00006

How Does Changing Pesticide Usage Over Time Affect Migrating Amphibians: A Case Study on the Use of Glyphosate-Based Herbicides in German Agriculture Over 20 Years

Gert Berger[1], Frieder Graef[1], Bernhard Pallut[2], Jörg Hoffmann[3], Carsten A. Brühl[4] and Norman Wagner[5]*

[1] Leibniz Centre for Agricultural Landscape Research, Müncheberg, Germany, [2] Formerly at Federal Research Centre for Cultivated Plants, Institute for Strategies and Technology Assessment, Julius Kühn-Institut, Kleinmachnow, Germany, [3] Federal Research Centre for Cultivated Plants, Institute for Strategies and Technology Assessment, Julius Kühn-Institut, Kleinmachnow, Germany, [4] Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany, [5] Department of Biogeography, Trier University, Trier, Germany

OPEN ACCESS

Edited by:
Enrique Martínez-Meyer, Universidad Nacional Autónoma de México, Mexico

Reviewed by:
Glen John Van Der Kraak, University of Guelph, Canada
Andres Garcia, Universidad Nacional Autónoma de México, Mexico

***Correspondence:**
Frieder Graef
fgraef@zalf.de

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *21 April 2016*
Accepted: *17 January 2018*
Published: *14 February 2018*

Citation:
Berger G, Graef F, Pallut B, Hoffmann J, Brühl CA and Wagner N (2018) How Does Changing Pesticide Usage Over Time Affect Migrating Amphibians: A Case Study on the Use of Glyphosate-Based Herbicides in German Agriculture Over 20 Years. Front. Environ. Sci. 6:6. doi: 10.3389/fenvs.2018.00006

Since its introduction in 1974, the use of glyphosate in agriculture has been continuously increasing; however, the application modes of this herbicide have been changing. Therefore, glyphosate-based herbicides can be used as an appropriate indicator for assessing how changes in pesticide application modes affect wild-living organisms in agricultural landscapes over time. Amphibians that migrate through arable fields may be exposed to the chemicals applied to field crops. Using data on the temporal coincidence of four amphibian populations with glyphosate applications from a three-year investigation in northeast Germany as well as data on the application of glyphosate to field crops in German agriculture over 20 years, we estimated the species-specific increasing rates of coincidence likelihoods during this period. The overall consumption of glyphosate used in German agriculture between 1992 and 2012 increased by a factor of 5.7, while the species-specific coincidence likelihood increased from 2.2 to 6.1, respectively. Our results reveal the highest increases in coincidence for both adult and juvenile great crested newt (*Triturus cristatus*) and fire-bellied toad (*Bombina bombina*). Adults and juveniles of moor frog (*Rana arvalis*) and adults of spadefoot toad (*Pelobates fuscus*) were subjected to moderate increases, with rates ranging from 3.2 to 3.6; in contrast, juvenile individuals of *P. fuscus* showed small increases. We suggest that the risk assessments of pesticide application (in this case, glyphosate) should not only consider the present use at the time of authorization but also consider changes in application modes over time that may lead to increases in potential exposure of non-target organisms, such as amphibians.

Keywords: amphibians, migration, glyphosate, application practice, temporal coincidence, crop fields

INTRODUCTION

One of the suggested major drivers for the global decline of amphibians is the intensification of agriculture and its associated activities (Collins and Storfer, 2003; Hayes et al., 2006; Boone et al., 2007; Mann et al., 2009; Brühl et al., 2013). In addition to the loss of habitats in agricultural areas, agrochemical pollution, which often interacts with other factors like climate change, UV-B radiation, emerging infectious diseases and alien species (Collins and Storfer, 2003; Stuart et al., 2008), is detrimental to amphibians (Mann et al., 2009). The impacts of agrochemicals on amphibians in water bodies (Xu and Oldham, 1997; Relyea, 2009; Biga and Blaustein, 2013) and on land (Oldham et al., 1997; Mann and Bidwell, 1999; Marco et al., 2001; Howe et al., 2004; Cauble and Wagner, 2005; Relyea, 2005; Bernal et al., 2009; Dinehart et al., 2009; Belden et al., 2010; Brühl et al., 2013) have been thoroughly studied and documented (Govindarajulu, 2008; Mann et al., 2009; Relyea, 2011; Wagner et al., 2013).

Glyphosate-based formulations have been shown to be toxic, especially during the aquatic life stages of amphibians, and often the commonly added surfactant POEA (polyethoxylated tallow amine) is mainly responsible for adverse effects (Giesy et al., 2000; Brausch and Smith, 2007; Brausch et al., 2007; Moore et al., 2012). Taking the particular risks of POEA into consideration, POEA-free glyphosate-based formulations have increasingly been used in German agriculture since 2013 (Rossberg, 2015a). The effects of glyphosate-based herbicides (hereafter referred to as GBH) on amphibians are formulation, species and life-stage specific and include osmotic instability, delayed or accelerated development, reduced size at metamorphosis, malformations, stress, and death (Wagner et al., 2013). Severe toxic effects caused by direct over-spraying of GBH on terrestrial juvenile stages of different anuran species have been revealed and documented in laboratory studies (Relyea, 2005; Bernal et al., 2009; Dinehart et al., 2009); however, scientific evidence on the toxicity of GBH on amphibians in field conditions is rare. We did not reflect the real impact of GBH on amphibian populations, and we did not consider interception by the crop canopy; thus, real field exposure was not studied. Rather, we used the increasing crop-specific usage of GBH in German agriculture over time and the temporal overlap of pesticide applications to crops with when amphibian populations are present in fields to determine the species-specific potential risk.

Globally, glyphosate (hereafter referred to as GLY) was introduced to agriculture in 1974 (BCPC, 2003) and was registered in Germany in 1975 (BVL, 2009). Between 1999 and 2008, the annual growth rate of its use in Germany was approximately 20% (Steinmann et al., 2012). GLY applications cover a wide range of crops and agronomic measures (Dill et al., 2010). Its modes of application, however, are currently changing. In the Americas and other parts of the world, the increase in the use of GLY correlates with the increase in the cultivation of genetically modified GLY-tolerant crops (Duke and Powles, 2008); however, in Germany, most genetically modified crops are still awaiting approval (Wagner and Lötters, 2013). Additionally, the present GLY usage in Germany is not only restricted to weed control but also promotes the entire production process, including reducing soil tillage and seedbed preparation, preventing erosion, controlling crop ripening (siccation) and harvesting, and managing stubble (Dill et al., 2010; Steinmann et al., 2012). These changing modes of herbicide application over time may alter the exposure risk of amphibians.

While the effects of GLY and GBH on larval amphibians have been well studied (see the review by Wagner et al., 2013 and more recent studies, e.g., Vincent and Davidson, 2015; Baier et al., 2016; Güngördü et al., 2016; Rissoli et al., 2016; Soloneski et al., 2016), there have been few studies on the effects of GBH on terrestrially active amphibians (oral exposure: McComb et al., 2008; direct over-spraying: Relyea, 2005; Bernal et al., 2009; Dinehart et al., 2009). Edge et al. (2011, 2013) exposed newly metamorphosed juveniles within the land-water transitional zone and found little or no effects on juvenile survival. Other studies exposed amphibians in terrestrial life-stages to GBH that was dissolved in water (Mann and Bidwell, 1999; Lajmanovich et al., 2015). Very little is known about the effects of GLY and its formulations on amphibians moving between breeding ponds and terrestrial habitats during non-breeding periods during the year (Relyea, 2005; Berger et al., 2013). Hence, it is important to analyze and quantify changes in the likelihood of the potential exposure of amphibian populations to GLY in crop fields over long periods. Berger et al. (2013) carried out an extensive field survey on the temporal coincidence of four typical amphibian species with GLY. Using expert estimations on the modes of GLY usage in agriculture over 20 years, we calculated the species- and age-level specific increases in the probability of temporal coincidence of amphibian populations.

We hypothesized that the overall increase in GLY consumption in German agriculture between 1992 and 2012 affected amphibian populations to various extents. The rates of increase of coincidence likelihood of the amphibian populations are different and are dependent on species and age level; thus, we can identify focal species that are particularly coincident in fields with GLY applications in German agriculture.

MATERIALS AND METHODS

Reference Year and Investigation Periods

Annual crop production and GLY sales for agriculture varied over the tenure of 20 years (i.e., 1992–2012). Therefore, we considered three average values for this period by considering the cultivation of arable crops × 1000 ha, which is abbreviated in this paper as Tha, and the GLY sales (t) for the periods of 1991–1993, 2001–2003, and 2011–2013; these data were used as a reference. These average values are referred to as investigation year 1992, 2002, and 2012, respectively. Further, based on a field survey that covered a wide range of agricultural farm situations throughout Germany and was valid for the reference year 2009, Steinmann et al. (2012) calculated the relative share of crop area treated with the three GLY application modes. The authors supported their calculated data by comparing them with the GLY sales and consumption in German agriculture during this reference year. All extrapolations and investigations of our work are linked to this reference year.

Glyphosate Sale and Usage in German Agriculture from 1988 to 2013

Statistical data on the sale and usage of GLY in German agriculture were available, though data were of various qualities. We derived data on GLY usage from 2009 onwards by analyzing the annual reports of BVL (Bundesamt für Verbraucherschutz und Landwirtschaft), which is the regulating authority for pesticides in Germany (BVL, 2014a,c). The consumption between 1995 and 2008 was derived from annual data on the sale of organophosphorus herbicides, which was specifically delivered by the German authority Federal Office of Consumer Protection and Food Safety (BVL, 2014b). Due to specific marketing rights of a single company during the period between 1988 and 1994, the BVL authority provided only an average value of the annual sale (BVL, 2014b). Applying the assessed trend function for the annual increase ($yc = 166.45x + 129.7$; for 1988: $x = 1$, and for 1994: $x = 7$), which was revealed by pre-analyses, we derived the annual values of the consumption of GLY from 1988 to 1994. We verified our estimations by visually analyzing the extrapolated values on the chart. The amount of GLY sold to professional users during the investigation years are based on the average sales from three consecutive years. Of the total estimated sales, 90% was used by farmers (Rossberg, 2015b), and the remaining 10% was used by other professionals belonging to horticulture, viniculture, railway companies and municipalities.

Expert Estimation and Validation of Glyphosate Usage Per Application Scheme and Crop

The usage schemes of GLY in agriculture for the investigation years 1992, 2002, and 2012 were jointly assessed by consensus of four experts with knowledge of GLY use in agriculture. First, the experts listed the changes in 1992, 2002, and 2012 as compared to the reference year 2009. For instance, (a) "most farmers did not apply glyphosate to winter barley stubbles in 1992" and (b) "due to increasing importance of reduced soil tillage to winter crops from 1992 to 2002 and to 2012, the stubble application on winter barley continuously increased." Second, these identified changes were translated into relative numbers. For instance, compared to 2009, the application areas in 1992, 2002, and 2012 were estimated to be 10% (factor of 0.1–2009), 70% (factor of 0.7–2009) and 110% (factor of 1.1–2009), respectively. Third, based on the numbers relative to 2009, we calculated the proportion of area where GLY was applied, considering all application schemes and crops for the identified study years. Multiplying this value by the total cultivation area of crops, we derived the area of crops treated with GLY for each investigation year and included the application modes. Finally, using the methodology of Steinmann et al. (2012), the application rate of GLY per hectare, and the application modes for all crops, we calculated the amount of GLY applied in the investigation years. Then, we calculated the total amount of GLY usage in German agriculture for each investigation year and compared it to the corresponding statistical data on GLY sales and consumption in German agriculture. When the deviance between the calculated values and the real values was less than 5%, we considered the values to be satisfactory; in contrast, higher deviations were used to iteratively adapt the estimates that the experts felt less confident about.

Field Data on the Temporal Coincidence of Amphibians with Glyphosate Applications to Crops

The quantitative field data available for the temporal coincidence of amphibian populations with the application of GLY on arable fields were provided by a field survey carried out between 2006 and 2008 in a study area located 50 km east of Berlin, Germany (Berger et al., 2013). This landscape has intensive agriculture use and is pond rich, so we analyzed four typically occurring amphibian species [fire-bellied toad (*Bombina bombina,* Linnaeus, 1761), moor frog (*Rana arvalis,* Nilsson, 1842), spadefoot toad (*Pelobates fuscus,* Laurenti, 1768) and northern crested newt (*Triturus cristatus,* Laurenti, 1768)] by using fence trapping during the annual migration periods. These amphibian species cover a wide range of different migration periods. Forty-nine drift fences, which consisted of 26 open, 10-m-long, cross-shaped fences, and 23 enclosures were installed between field machinery tramlines. The cross-shaped fences were regularly distributed in a 400 × 400 m grid to record amphibian migration activity in fields, and they encircled biotopes (i.e., wood lots, small water bodies) located at the edges of fields or completely within fields. Depending on the direction of migration, either the inner or outer traps were analyzed. Captured individuals were released 10–15 m from the opposite site of the fence.

We included three different application modes (e.g., pre-sowing or pre-emerging application in spring, siccation in summer, and pre-harvesting and stubble management in late summer/autumn prior to crop sowing) and six major field crops (maize, triticale, winter barley, winter rape, winter rye and winter wheat); additionally, we used the four amphibian species listed above and their age-specific coincidence values for two DT_{50} values of GLY (i.e., 12 and 47 days) separately. These values allowed the derivation of important indicators for the co-occurrence and potential exposure of amphibian populations (Berger et al., 2013). The three GLY application modes used in Steinmann et al. (2012) partially differed from the modes applied by Berger et al. (2013). Thus, each of our crop GLY application modes was adjusted to match the "Steinmann system." The use of the two DT_{50} levels ("Dissipation Time." i.e., the half-life of the active ingredient of GLY in soils) as well as the average of the single coincidence values for each field was done according to Berger et al. (2013). The DT_{50} values of GLY ranged from 2 to 197 days, with a typical field half-life of 47 days (Miller et al., 2010). The DT_{50} estimates were available for the POEA surfactant only (i.e., 21–41 days; but not for other substances added to the formulation; Giesy et al., 2000). Hence, the DT_{50} values for GLY must serve as proxies for GBH. We calculated and applied coincidence values for each GLY application scheme and for each crop as averages of the two DT_{50} levels.

Calculating the Species and Age-Class Specific Changes of Coincidence Values

Changes in the coincidence likelihood between amphibian populations and the application of GLY to field crops depend on (a) the increase in GLY application area per application scheme over time and (b) the species and their age-level-specific share of the amphibian populations. To find a species and age-specific indicator value suitable for explaining changes over years, we multiplied the coincidence values and the GLY application area, which enabled the calculation of the coincidence likelihood Equation (1).

$$\mathrm{CLiR}_{(n+1)\text{ to }n} = \frac{\mathrm{CLA}_{\mathrm{year(n+1)}} \times \mathrm{ApplRate}_{\mathrm{apptype} \times \mathrm{year(n+1)}} \times \mathrm{CV}_{\mathrm{apptype} \times \mathrm{spec} \times \mathrm{age}}}{\mathrm{CLA}_{\mathrm{year(n)}} \times \mathrm{ApplRate}_{\mathrm{apptype} \times \mathrm{year(n)}} \times \mathrm{CV}_{\mathrm{apptype} \times \mathrm{spec} \times \mathrm{age}}} \quad (1)$$

CLiR (n+1) to n: coincidence likelihood ratio between one year (n) and the following (n+1). $\mathrm{CLA}_{\mathrm{year}}$ …: crop land area per year (i.e., average values of three consecutive years: 1992: 1991–1993; 2002: 2001–2003; 2012: 2011–2013, [Tha]); $\mathrm{ApplRate}_{\mathrm{appltype} \times \mathrm{year}}$ …: GLY application rate of crop land per year and period [%]; $\mathrm{CV}_{\mathrm{appltype} \times \mathrm{spec} \times \mathrm{age}}$: average coincidence values of two migration periods and two DT_{50} values for 4 amphibian species and 2 age classes [%].

The ratio of the obtained products indicate the likelihood of an increase in coincidence between periods. The analyses were performed for the four amphibian species, their two age levels, and the two migration periods (i.e., from and to ponds).

RESULTS

Overall Glyphosate Sales and Consumption in German Agriculture Over the Last 20 Years

In Germany, GLY usage by professional applicators (e.g., agriculture, horticulture, railway companies, municipalities) increased from 956 t of active substance in 1992 to 5415 t of active substance in 2012 (BVL, 2014a). Thus, the consumption in 2012 was 5.7 times higher than the consumption 20 years ago. In 2002, approximately 3740 t GLY was applied, corresponding to a 3.9-fold increase compared to 1992 and a 1.5-fold increase from 2002 to 2012. In terms of agricultural use, we assumed 90% of the total consumption was by professionals (860, 3.365 and 4.873 t, respectively), leading to the same increase in values between periods.

Expert Estimations on the Relative Changes of Glyphosate Application Modes to Arable Crops Over 20 Years

The factors relative to the GLY application in 2009, as estimated by experts, varied between the crops and periods (**Figure 1**). Except for maize, which had a factor of 0.5, the pre-sowing applications to all other crops in 1992 were estimated to be approximately 0.1 of the application value of 2009. In both 2002 and 2012, the application values for this crop surpassed the application value of 2009. In 2002, the other range of crop factors (relative to 2009) varied from 0.7 to 0.8, and this value was 1.0 in 2012. For pre-sowing application, compared to the values from 2009, we estimated an additional increase of 1.2 for winter wheat and an increase of 1.1 for both oilseed rape and corn.

Calculated Area of Glyphosate Applied on Arable Land and GLY Consumption in German Agriculture

The total area of GLY application in German agriculture, including grassland (not shown), was ≈740 Tha in 1992; ≈2.930 Tha in 2002; and ≈4.250 Tha in 2012. Based on the area of GLY application and the estimated application rates per crop, the consumption of GLY in agriculture was 845 t, 3.340 t, and 4.865 t for 1992, 2002, and 2012, respectively. These values differed between −0.2 and −1.7% from the statistically grounded sale of GLY to agricultural buyers.

The areas with most of the 6 main crops, i.e., winter wheat (wwt), silage maize (mze), oilseed rape (wra), winter barley (wbl) and winter rye/triticale (wry/trc), considered for amphibian coincidence analyses increased over 20 years. The increase from 1992 to 2012 ranged from 1.3 to 1.6, though the value for winter barley fell outside this range. The area of maize increased the most between 2002 and 2012, with a rate of 1.8 during this 10-year period.

The application area of these 6 crops increased from 663 Tha in 1992 to 2286 Tha in 2002 and to 3461 Tha in 2012, encompassing between 81 and 94% of the total area where GLY was applied on arable land. The GLY application area increased from by factor 5.2 between 1992 and 2012, by a factor of 3.4 between 1992 and 2002, and by a factor of 1.5 between 2002 and 2012. The GLY application area was largely different between periods and plants (**Figure 2**). Wwt, wra, and wbl covered large areas with the steepest increases occurring between 1992 and 2002. This contrasts with the GLY application area of mze, which particularly increased between 2002 and 2012, but never reached the application area of wwt, wra and wbl.

Allocation of Glyphosate to Application Modes of Crops Considered for Amphibian Coincidence

Except for pre-harvesting applications for mze, the GLY application areas for all other crops with different application modes increased. The average rate of increase of 9.1 was exceeded for wwt, wra, and rye/trc for all three applications modes. For pre-sowing (ps/pe) and pre-harvesting (ph) in wwt and for ps/pe in wra, we found a 16-fold increase in rates. When comparing the rates of increase between 1992 and 2002 with those between 2002 and 2012, we mostly found higher values during the first period. As shown for ps/pe and ph in wry/trc, the values were as much as 8-fold higher. With respect to the size of application area, stubble application (sa) of wra was always ranked first during the entire 20-year period, though ranks 2 through 4 occasionally changed over time. From 2002 to 2012, we found the same combinations of crops and application modes in these rank positions, albeit in

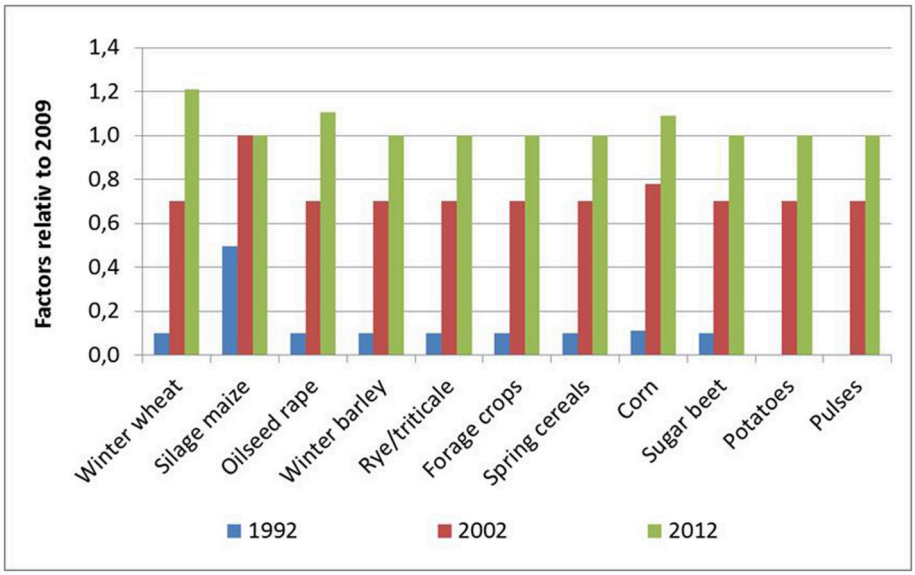


FIGURE 1 | Expert estimations of the relative deviances from the reference year 2009 (Steinmann et al., 2012) on the amount of GLY applied in pre-sowing applications of GBH to arable crops in the three investigation years.

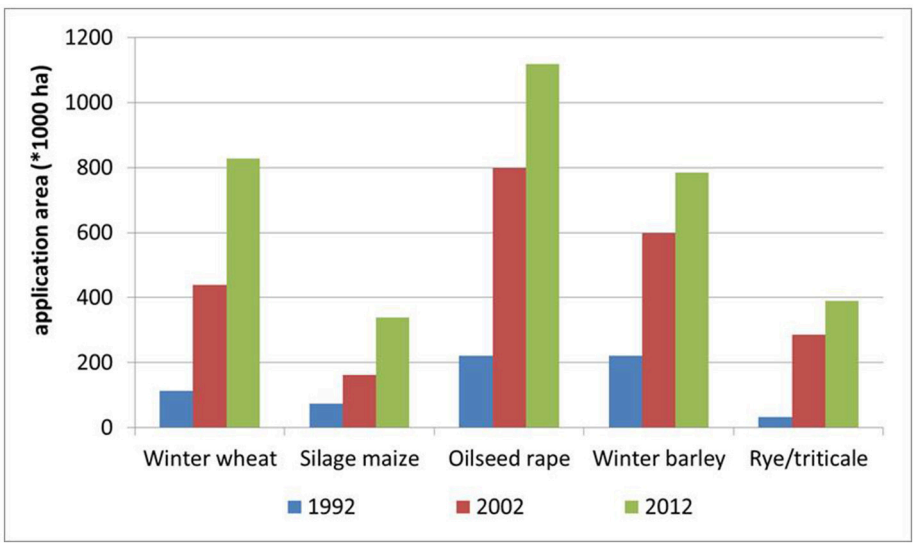


FIGURE 2 | Total GLY application area for each crop considered for amphibian coincidence during the three investigation periods over 20 years.

different orders. In 1992, ps/pe in mze was in rank 4, and ph in wbl was in rank 2. Both lost these ranked positions in following years.

Amphibian-Specific Increase of Coincidence Likelihood

The rise of GLY in agriculture during the 20-year period led to increasing rates of coincidence likelihood of amphibians from 2.2 to 6.1 (**Table 1**). We found the amphibian coincidence likelihood increased by an average of a factor of 4.1 between 1992 and 2012, 2.6 between 1992 and 2002, and 1.5 between 2002 and 2012. The coincidence likelihood rates of increase for adults of all four species migrating to ponds were about one-third lower than the average coincidence likelihoods observed between 1992 and 2002. The same was found for adults of *P. fuscus* and for juveniles of *R. arvalis* and *P. fuscus* that migrated from ponds. In contrast, adults and juveniles of *T. cristatus* and *B. bombina* largely exceeded the average increases. Their coincidence likelihoods were about one-half higher than the averages and almost twice as high than the other amphibian groups. Between 2002 and 2012, the increases in coincidence likelihoods for the investigated amphibian groups were different

TABLE 1 | Cumulative product values and rate of increase according to migration, age and species (bold* indicates values above mean).

Migration	Age	Species	Cumulative product values (GLY application area * coincident population share)			Rate of increase		
		Period	1992	2002	2012	1992–2002	2002–2012	1992–2012
To ponds	Adults	*R. arvalis*	1.5334	2.7762	4.9398	1.8	**1.8***	3.2
		T. cristatus	2.4904	4.5087	8.0225	1.8	**1.8***	3.2
		P. fuscus	1.4154	2.5624	4.5595	1.8	**1.8***	3.2
		B. bombina	4.2799	7.7486	13.7875	1.8	**1.8***	3.2
From ponds	Adults	*T. cristatus*	13.2085	51.5473	80.2051	**3.9***	1.6	**6.1***
		P. fuscus	2.543, 5	4.6048	8.1936	1.8	**1.8***	3.2
		B. bombina	13.9743	52.0846	78.7004	**3.7***	1.5	**5.6***
	Juveniles	*Rana arvalis*	8.0988	20.9970	29.5561	**2.6***	1.4	3.6
		Triturus cristatus	14.8296	60.0357	89.6121	**4.0***	1.5	**6.0***
		Pelobates fuscus	10.5586	17.3677	23.4559	1.6	1.4	2.2
		Bombina bombina	7.3688	30.4893	42.7882	**4.1***	1.4	**5.8***
				Mean		2.6	1.6	4.1

than the values from the preceding decade (**Table 1**). The rate of increase of coincidence likelihoods for adults of all species migrating into ponds and for adults of *P. fuscus* leaving ponds were above average. However, for all juveniles and for adults of *T. cristatus* and *B. bombina*, we found increases of coincidence likelihoods that were equal or lower than the average value.

The increases of coincidence likelihoods for the entire investigation period (i.e., 1992–2012) were similar to the changes between 1992 and 2002, though the changes were at various extents (**Table 1**). With almost a 6-fold rate of increase, we found the highest values for adults and juveniles of *T. cristatus*; in contrast, the coincidence likelihood of juveniles of *P. fuscus* increased only by a factor of 2.2.

The increases in coincidence of likelihoods for species and age levels were found to vary between 1.4 and 6.1. For *T. cristatus* and *B. bombina*, the highest increases were recorded between 1992 and 2002, though we found higher coincidence likelihood increases for adults of *P. fuscus* and *R. arvalis* during the second period. Generally, for the former two species, we found profoundly lower rates of increase compared to the others, and, except for *P. fuscus,* we found higher increases for juveniles than for adults over the 20-year period.

DISCUSSION

To date, there are no existing methodologies or studies on long-term changes in GLY application modes and the linkage to amphibian migration. We used coincidence values from an extensive three-year investigation on both the temporal application of GLY to crops and the share of migrating amphibian populations in fields. Based on that, we partially refer to the potential exposure while acknowledging that the interception by the plant canopy and the specific field conditions were not considered but should be subjects of further research. For example, soil or litter remarkably reduced the adverse effects on over-sprayed amphibians in terrestrial life stages in two laboratory studies (Bernal et al., 2009; Dinehart et al., 2009). However, laboratory data show that the active ingredient (GLY) of the GBH permeates the skin of edible frogs (*Pelophylax* kl. *esculentus*) 26-times faster than it permeates pig skin (Quaranta et al., 2009). The acute and chronic effects of GBH uptake in the terrestrial life stages of amphibians are documented. For example, 79% of tested juvenile anurans died within one day after direct over-spraying with a commercial GBH at recommended application rates in North America (Relyea, 2005). Additionally, 30% of individuals of several tested anuran species died within 24 h after direct over-spraying at usual application rates of a GBH used in coca plant eradication in Colombia (Bernal et al., 2009). Based on results from studies using larvae, different GBH formulations pose different risks for terrestrial amphibian life stages. Depending on the tested formulation, mortality ranged from 0 to 80% in over-sprayed Great Plains toads (*Bufo cognatus*) and New Mexico spadefoots (*Spea multiplicata*) (Dinehart et al., 2009). Hence, the fast absorption of GLY should not be the most important cause of observed effects, as adjuvants are usually mainly responsible instead (Wagner et al., 2013). However, to date, there are no data on absorption time and rates of added substances (like POEA surfactants) in amphibians. From the tropics, there is also an anecdotal report that caecilians with burn-like wounds were found 5–7 h after Roundup applications in a tea plantation in Sri Lanka (de Silva, 2009). Finally, sublethal low doses of a GBH induced neurotoxicity, oxidative stress and immunological depression in exposed Argentine toads (*Rhinella arenarum*) (Lajmanovich et al., 2015).

The coincidence values used in our calculations are based on data from a single investigation area in northeast Germany. Though the field management and species coincidence values may vary between regions, we presume that the principal linkage between the timing of GLY applications to crops and amphibian behavior is similar on large spatial scales. It is very likely that the species and age levels of amphibians that were more coincident

to glyphosate applications in our investigation area will be more coincident in other regions too. This is because both the migration modes of the species (i.e., early or late migrating species) and the crop cultivation systems with respect to GLY applications are widely similar. Additionally, both the timing of agricultural cultivation and the timing of amphibian activity are driven by climate and weather conditions and may lead to a uniform time shift (Lötters et al., 2014). Thus, we are confident the observed trends are valid for arable regions in the northern lowlands of Germany where glyphosate is applied and amphibians are present. We validated the expert estimates on GLY usage in German agriculture using the statistical data on GLY agricultural consumption. Since the official statistical land-use data of Germany includes an error of 2–10% (Steinmann et al., 2012), we considered deviances lower than 2% from the real sales to be appropriate.

Our results indicate that generalizing the increase in sales and consumption of pesticides, in this case GLY, in agriculture is inappropriate for characterizing the potential exposure of specific organisms. While the overall GLY consumption increased by a factor of 5.7 between 1992 and 2012, the rates of species coincidence likelihoods varied widely. Both the crop-specific GLY use among various applications and the changes in the cultivation area of crops over time increased the coincidence likelihoods, though these changes were at different extents. With a 16-fold increase in GLY application area, the application mode of pre-sowing to wwt showed highest change. Since there were no scientifically supported coincidence values for this application mode, its impact on the increase of coincidence likelihood of species was not analyzed.

We found the highest ranks among the area of GLY application to crops and periods for the following two modes, pre-sowing/pre-emerging and stubble applications. Both are closely related to reduced or conservation tillage without plowing. Over time, glyphosate has become the backbone of no-till agriculture (Duke and Powles, 2008; Yadav et al., 2013). Together with the control of green biomass (volunteers and weeds, *Agropyron repens* in particular), these are considered to be the main drivers of GLY application (Raubuch and Schieferstein, 2002; Nail et al., 2007). In German crop rotations, rewardable winter cereals, such as winter wheat, winter barley and triticale, often follow winter rape (Steinmann and Dobers, 2013), and in accordance to our findings, field cultivation is often conducted in combination with GLY application. In 2009, winter rape was considered the major sink of GLY in Germany (Steinmann et al., 2012). This was confirmed in our study for the years 2002 and 2012. In 2009/2010, approximately 39% of German arable land was cultivated by reduced tillage, covering approximately 4.469 Mio ha, underlining the importance of this type of soil management (Destatis, 2011).

For many years, pre-harvesting application (siccation) was common in winter barley. With the objectives of cleaning weeds and volunteers from the crop, controlling the timing of harvest, and better adjusting harvest technique siccation, the use of GLY has become increasingly prominent in other arable crops too (Cook et al., 2010). In terms of winter wheat, our analyses indicate up to 17-fold increases in GLY application areas over the 20-year period, with the highest application level in 2012. Nonetheless, even in 2012, the total area of pre-harvesting application in Germany did not exceed 5% of the arable land. Thus, this was still far below what is found in the UK, where 40–80% of cereals and oilseed rape are regularly treated for siccation (Cook et al., 2010). Starting in 2014, pre-harvesting siccation regulations changed; for instance, the application depends on the specific crop canopy conditions (BVL, 2014a). This may lead to a reduced application area.

The change in crop area over time is another important driver that affects the increase in GLY usage in agriculture. Some economically profitable crops, such as winter wheat, oilseed rape and silage maize, have increased, leading to narrower crop rotations and fewer crop species (Steinmann and Dobers, 2013; Destatis, 2015). Reducing the number of crops leads to more intensive peak-periods for conducting field cultivation and increases demands for higher working rates. For stubble cultivation, GLY application appears to be appropriate (Nail et al., 2007; Steinmann et al., 2012). A small number of crops linked with reduced tillage and a monotonic herbicide application scheme with active ingredients that are based on similar effective mechanisms may also cause resistant weeds that are hard to control (Weedscience, 2015). Higher rates and an increase in the application of herbicides, including GLY, are then necessary (Beckie, 2006). Narrow crop rotations may promote plant pests and diseases (Steinmann and Dobers, 2013). Controlling the green bridge between two successive crops to prevent the spread of pests or disease is particularly essential and often typically done using GLY.

With respect to the year 2009, Steinmann et al. (2012) characterized GLY applications "as a routine application facilitating many agronomical purposes." GLY emerged as a weed control instrument and shifted to a multifunctional agronomical tool that replaced traditional practices; it can also be used to save labor and machinery input. Our results confirmed the author's conclusion for Germany and indicated what today's wide GLY usage (for many agronomic purposes) may mean for wild-living organisms. Together with its worldwide use in genetically modified crops, GLY has become the best-selling herbicide in many countries and may be used as a sole herbicide (Duke and Powles, 2008). In Asia, for instance, there are many countries that are rich in amphibian diversity but are also experiencing rapid growth in agricultural production, and the consumption of glyphosate and other herbicides is increasing. Though it is known that, in most cases, applications of agrochemicals overlap with the breeding activities of amphibians in agro-ecosystems on the Indian subcontinent (Hegde and Krishnamurthy, 2014), there is a complete dearth of information on the rate of coincidence of migrating amphibian populations under this agro-chemical environment (Mann et al., 2009). Therefore, the present work could be a guideline for those countries where plant protection products are increasingly being used and are leading to species-specific increases in potential exposure of amphibian populations.

The semi-steep increase in coincidence likelihoods with amphibians indicates the need for further research. Specifically, since European endangered and protected species, such as *T.*

cristatus and *B. bombina* (EU, 1992), are experiencing increasing potential exposure, a deeper scientific look into the potential and real impacts of GBH applications on these protected species is undoubtedly necessary.

The exposure potential we considered in this study is based on migrating terrestrial life stages of amphibian populations. In the northeastern plains of Germany, approximately 25% of breeding ponds are located within crop fields, and another 25% of ponds are directly adjacent to field edges (Berger et al., 2011). The reported increase in the areas where GLY is applied and the changes in application schemes and crops over the last 20 years not only meet amphibians on land but also increase the risk of breeding ponds being contaminated by GLY. This may entail adverse effects on eggs and larvae (Relyea, 2005; Wagner et al., 2013). Additionally, indirect adverse effects on amphibians that are related to GLY may occur. For instance, GLY completely controls and removes green biomass, and it interferes with regulative interactions and food webs and likely changes food provisions for a wide range of wild-living organisms, including amphibians (Govindarajulu, 2008; Geiger et al., 2010; Pérez et al., 2011; Jahn et al., 2014).

CONCLUSIONS

Glyphosate usage in German agriculture has changed considerably over the 20-year study period. Our analysis investigated how wild-living amphibians present in fields were likely to be increasingly exposed to GLY during this time. For the environmental risk assessment and regulation of plant protection products, we advocate considering not only the state and usage of pesticides at the time of their authorization but also the changes in application schemes and extents over time. Thus, we recommend conducting periodical reassessments of environmental risks—not only on the toxicity to organisms but also on the present and practical usage modes in agriculture that affect potential exposure patterns.

AUTHOR CONTRIBUTIONS

GB: Main organisor of the study, data analysis and manuscript writing. FG: Major expertise contributor, data analysis, and manuscript writing. BP: Major expertise contributor, data analysis, and manuscript writing. JH: Expertise contributor, data provision and analysis, and manuscript writing. CB: Expertise contributor, data generation, provision and analysis, and manuscript writing. NW: Expertise contributor, data provision and analysis, and manuscript writing.

FUNDING

This project was funded by the Umweltbundesamt, Germany under the award number FKZ: 3709 65 421.

ACKNOWLEDGMENTS

We thank Dietmar Rossberg from the Federal Research Centre for Cultivated Plants, Kleinmachnow, Germany for contributing to the expert estimations and Horst-Henning Steinmann for additional explanations on the paper that were fundamental for our work. The Federal Office for Consumer Protection and Food Safety, and Mirijam Seng in particular, provided additional data on the GLY sales in Germany. The authors also thank Ulrich Stachow, Harald Kaechele, and Peter Zander for their comments on the manuscript.

REFERENCES

Baier, F., Jedinger, M., Gruber, E., and Zaller, J. G. (2016). Temperature seems to be an important factor when assessing effects of a glyphosate-based herbicide on egg and tadpole growth of Common toads (*Bufo bufo*; Amphibia L.). *Front. Environ. Sci.* 4:51. doi: 10.3389/fenvs.2016.00051

BCPC (2003). Pesticide Manual. British Crop Protection Council. 13. Auflage 2003, 514 page., S. 514: "Commercialisation: History: Herbicidal activity reported by D.D. Baird et al. (Proc. North Cent. Weed Control Conf., 1976, 26, 64). The isopropylamine, sodium and ammonium salts introduced by Monsanto Co. in 1974; the trimesium (trimethylsulfonium) salt introduced in Spain (1989) by ICI Agrochemicals (now Syngenta AG). Patents US 3799758 (to Monsanto); EP 53871; US 4315765 (both to ICI)"

Beckie, H. (2006). Herbicide-resistant weeds: managent tactics and practices. *Weed Technol.* 20, 793–814. doi: 10.1614/WT-05-084R1.1

Belden, J., McMurry, S., Smith, L., and Reilley, P. (2010). Acute toxicity of fungicide formulations to amphibians at environmentally relevant concentrations. *Environ. Toxicol. Chem.* 29, 2477–2480. doi: 10.1002/etc.297

Berger, G., Graef, F., and Pfeffer, H. (2013). Glyphosate applications on arable fields considerably coincide with migrating amphibians. *Sci. Rep.* 3:2622. doi: 10.1038/srep02622

Berger, G., Pfeffer, H., and Kalettka, T. (eds). (2011). *Amphibienschutz in Kleingewässerreichen Ackerbaugebieten (Conservation of Amphibians in Agricultural Landscapes Rich in Small Water Bodies).* Rangsdorf: Natur & Text.

Bernal, M. H., Solomon, K. R., and Carrasquilla, G. (2009). Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. *J. Toxicol. Env. Health A* 72, 966–973. doi: 10.1080/15287390902929717

Biga, L. M., and Blaustein, A. R. (2013). Variations in lethal and sublethal effects of cypermethrin among aquatic stages and species of anuran amphibians. *Environ. Toxicol. Chem.* 32, 2855–2860. doi: 10.1002/etc.2379

Boone, M. D., Cowman, D., Davidson, C., Hayes, T., Hopkins, W., Relyea, R. A., et al. (2007). "Evaluating the role of environmental contamination in amphibian population declines," in *Amphibian Conservation Action Plan.* eds C. Gascon, J. P. Collins, R. D. Moore, D. R. Church, J. E. McKay, and J. R. I. Mendelson (Gland and Cambridge: IUCN/SSC Amphibian Specialist Group), 32–36.

Brausch, J. M., Beall, B., and Smith, P. N. (2007). Acute and sub-lethal toxicity of three polyethoxylated alkylamine surfactant formulations to *Daphnia magna*. *Bull. Environ. Contam. Tox.* 78, 510–514. doi: 10.1007/s00128-007-9091-0

Brausch, J. M., and Smith, P. N. (2007). Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, *Thamnocephalus platyurus*. *Arch. Environ. Contam. Toxicol.* 52, 217–221. doi: 10.1007/s00244-006-0151-y

Brühl, C. A., Schmidt, T., Pieper, S., and Alscher, A. (2013). Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Sci. Rep.* 3:1135. doi: 10.1038/srep01135

BVL (2009). *Berichte zu Pflanzenschutzmitteln. Wirkstoffe in Pflanzenschutzmitteln, Zulassungshistorie und Regelungen der Pflanzenschutz-Anwendungsverordnung.* Available online at: http://www.bvl.bund.de/

SharedDocs/Downloads/04_Pflanzenschutzmittel/bericht_WirkstoffeInPSM_2009.html?nn=1401286 (Accessed Dec 16, 2014)

BVL (2014c). *Sales of Plant Protection Products in Germany. Statistics According to § 19 Plant Protection Act (PflSchG) for Years 2009-2013*. Federal Office for Consumer Protection and Food Safety Braunschweig. Available online at: http://www.bvl.bund.de/DE/04_Pflanzenschutzmittel/01_Aufgaben/02_ZulassungPSM/03_PSMInlandsabsatzExport/psm_PSMInlandsabsatzExport_node.html (Accessed Dec 15, 2014)

BVL (2014a). *Application Rules of Glyphosate, 21.05.2014*. Available online at: http://www.bvl.bund.de/DE/04_Pflanzenschutzmittel/05_Fachmeldungen/2014/2014_05_21_Fa_Neue_Anwendung_Glyphosat.html?nn=1400938 (Accessed Nov 11, 2015)

BVL (2014b). *Sale of Organo-Phosphorous Herbicide Active Substances (Glyphosate and Glyphosinate) for 1988–2008 in Germany*. Braunschweig: Federal Office for Consumer Protection and Food Safety.

Cauble, K., and Wagner, R. S. (2005). Sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development. *Bull. Environ. Contam. Tox.* 75, 429–435. doi: 10.1007/s00128-005-0771-3

Collins, J. P., and Storfer, A. (2003). Global amphibian declines: sorting the hypotheses. *Divers. Distrib*. 9, 89–98. doi: 10.1046/j.1472-4642.2003.00012.x

Cook, S., Wynn, S., and Clarke, J. (2010). Glyphosate - a necessary herbicide. How valuable is glyphosate to UK agriculture and environment? *Outlooks Pest Manag.* 21, 280–283.

de Silva, A. (2009). *The Incidence and Pattern of Malformations, Abnormalities, Injuries, and Parasitic Infection of Amphibians in Sri Lanka (Preliminary Findings)*. Final Report. Amphibian Specialists Group, Washington DC.

Destatis (2011). *Land- und Forstwirtschaft, Fischerei. Bodenbearbeitung, Bewässerung, Landschaftselemente. Erhebung über Landwirtschaftliche Produktionsmethoden (ELPM), 2010. Fachserie 3, Heft 5*. Wiesbaden: Statistisches Bundesamt.

Destatis (2015). Mais https://www.destatis.de/DE/ZahlenFakten/WirtschaftsbereicheLandForstwirtschaftFischerei/FeldfruechteGruenland/Tabellen/FeldfruechteZeitreihe.html (Accessed Nov 02, 2015)

Dill, G. M., Sammons, R. D., Feng, P. C. C., Kohn, F., Kretzmer, K., Mehrsheikh, A., et al. (2010). "Glyphosate: discovery, development, applications, and properties," in Glyphosate Resistance in Crops and Weeds, ed V. K. Nandula (Hoboken, NJ: John Wiley & Sons), 1–33.

Dinehart, S. K., Smith, L. M., McMurry, S. T., Anderson, T. A., Smith, P. N., and Haukos, D. A. (2009). Toxicity of a glufosinate- and several glyphosate based herbicides to juvenile amphibians from the Southern High Plains, USA. *Sci. Total Environ.* 407, 1065–1071. doi: 10.1016/j.scitotenv.2008.10.010

Duke, S. O., and Powles, S. B. (2008). Glyphosate: a once-in-a-century herbicide. *Pest Manag. Sci.* 64, 319–325. doi: 10.1002/ps.1518

Edge, C. B., Gahl, M. K., Pauli, B. D., Thompson, D. G., and Houlahan, J. E. (2011). Exposure of juvenile green frogs (*Lithobates clamitans*) in littoral enclosures to a glyphosate-based herbicide. *Ecotoxicol. Environ. Saf.* 74, 1363–1369. doi: 10.1016/j.ecoenv.2011.04.020

Edge, C. B., Gahl, M. K., Thompson, D. G., and Houlahan, J. E. (2013). Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and *Batrachochytrium dendrobatidis*. *Sci. Total Environ*. 444, 145–152. doi: 10.1016/j.scitotenv.2012.11.045

EU (1992). Council directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. *Off. J.* 206, 7–50.

Geiger, F., Bengtsson, J., Berendse, F., Wolfgang, W. W., Emmerson, M., Morales, M. B., et al. (2010). Persistent Negative Effects of Pesticides on Biodiversity and Biological Control Potential on European Farmland. *Basic Appl. Ecol.* 11, 97–105. doi: 10.1016/j.baae.2009.12.001

Giesy, J. P., Dobson, S., and Solomon, K. R. (2000). Ecotoxicological risk assessment for Roundup herbicide. *Rev Environ Contam Toxicol* 167, 35–120. doi: 10.1007/978-1-4612-1156-3_2

Govindarajulu, P. P. (2008). *Literature Review of Impacts of Glyphosate Herbicide on Amphibians: What Risks can the Silvicultural Use of this Herbicide Pose for Amphibians in B.C.?* Wildlife Report No. R-28 B.C. Ministry of Environment, Victoria, BC.

Güngördü, A., Uçkun, M., and Yologlu, E. (2016). Integrated assessment of biochemical markers in premetamorphic tadpoles of three amphibian species exposed to glyphosate-and methidathion-based pesticides in single and combination forms. *Chemosphere* 144, 2024–2035. doi: 10.1016/j.chemosphere.2015.10.125

Hayes, T. B., Case, P., Chul, S., Chung, D., Haeffele, C., Haston, K., et al. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environ. Health Perspect*. 114, 40–50. doi: 10.1289/ehp.8051

Hegde, G., and Krishnamurthy, S. V. (2014). Analysis of health status of the frog Fejervarya limnocharis (Anura: Ranidae) living in rice paddy fields of Western Ghats, using body condition factor and AChE content. *Ecotoxicol. Environ. Contaminat*. 9, 69–76. doi: 10.5132/eec.2014.01.009

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., and Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environ. Toxicol. Chem.* 23, 1928–1938. doi: 10.1897/03-71

Jahn, T., Hoetker, H., Oppermann, R., Bleil, R., and Vele, L. (2014). *Protection of Biodiversity of Free Living Birds and Mammals in Respect of the Effects of Pesticides*. Umweltbundesamt. Available online at: https://www.umweltbundesamt.de/sites/default/files/medien/378/publikationen/texte_30_2014_protection_of_biodiversity.pdf

Lajmanovich, R. C., Attademo, A. M., Simoniello, M. F., Poletta, G. L., Junges, C. M., Peltzer, P. M., et al. (2015). Harmful Effects of the Dermal Intake of Commercial Formulations Containing Chlorpyrifos, 2, 4-,D, and Glyphosate on the Common Toad *Rhinella arenarum* (Anura: Bufonidae). *Water Air Soil Pollut.* 226:427. doi: 10.1007/s11270-015-2695-9

Lötters, S., Filz, K. J., Wagner, N., Schmidt, B. R., Emmerling, C., and Veith, M. (2014). Hypothesizing if responses to climate change affect herbicide exposure risk for amphibians. *Environ. Sci. Europe* 26:31. doi: 10.1186/s12302-014-0031-4

Mann, R. M., and Bidwell, J. R. (1999). The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Arch. Environ. Contam. Toxicol*. 36, 193–199. doi: 10.1007/s002449900460

Mann, R. M., Hyne, R. V., Choung, C. B., and Wilson, S. P. (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environ. Pollut*. 157, 2903–2927. doi: 10.1016/j.envpol.2009.05.015

Marco, A., Cash, D., Belden, L. K., and Blaustein, A. R. (2001). Sensitivity to urea fertilizationin three amphibian species. *Arch. Environ. Contam. Toxicol*. 40, 406–409. doi: 10.1007/s002440010190

McComb, B. C., Curtis, L., Chambers, C. L., Newton, M., and Bentson, K. (2008). Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. *Environ. Sci. Pollut. Res.* 15, 266-272. doi: 10.1065/espr2007.07.437

Miller, A., Gervais, J. A., Luukinen, B., Buhl, K., and Stone, D. (2010). *Glyphosate Technical Fact Sheet; National Pesticide Information Center, Oregon State University Extension Services*. Available online at: http://npic.orst.edu/factsheets/glyphotech.pdf (Accessed Dec 05, 2016).

Moore, L. J., Fuentes, L., Rodgers, J. H., Bowerman, W. W., Yarrow, G. K., and Chao, W. Y. (2012). Relative toxicity of the components of the original formulation of Roundup to five North American anurans. *Ecotoxicol. Environ. Saf.* 78, 128–133. doi: 10.1016/j.ecoenv.2011.11.025

Nail, E. L., Young, D. L., and Schillinger, W. F. (2007). Diesel and glyphosate price changes benefit the economics of conservation tillage versus traditional. *Tillage. Soil Till. Res*. 94, 321–327. doi: 10.1016/j.still.2006.08.007

Oldham, R. S., Latham, D. M., Hilton Brown, D., Towns, M., Cooke, A. S., and Burn, A. (1997).The effect of ammonium nitrate fertiliser on frog (*Rana temporaria*) survival. *Agric. Ecosyst. Environ*. 61, 69–74. doi: 10.1016/S0167-8809(96)01095-X

Pérez, G. L., Vera, M. S., and Miranda, L. (2011). *Effects of Herbicide Glyphosate and Glyphosate-Based Formulations on Aquatic Ecosystems, Herbicides and Environment, Dr Andreas Kortekamp (Ed.), ISBN: 978-953-307-476-4*, InTech, Available online at: http://www.intechopen.com/books/herbicides-andenvironment/effects-of-herbicide-glyphosate-and-glyphosate-based-formulations-on-aquatic-ecosystems

Quaranta, A., Bellantuono, V., Cassano, G., and Lippe, C. (2009). Why amphibians are more sensitive than mammals to xenobiotics. *PLoS ONE* 4:e7699. doi: 10.1371/journal.pone.0007699

Raubuch, M., and Schieferstein, B. (2002): *Ökologische und ökosystemanalytische Ansätze für das Monitoring von gentechnisch veränderten Organismen*. Berlin: Umweltbundesamt.

Relyea, R. A. (2005). The lethal impact of Roundup on aquatic and terrestrial amphibians. *Ecol. Applic.* 15, 1118–1124. doi: 10.1890/04-1291

Relyea, R. A. (2009). A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. *Oecologia* 159, 363–376. doi: 10.1007/s00442-008-1213-9

Relyea, R. A. (2011). "Amphibians are not ready for Roundup®," in *Wildlife Ecotoxicology,* Vol 3 *Emerging Topics in Ecotoxicology*, eds J. E. Elliott, C.A. Bishop, and C. A. Morrissey (New York, NY: Springer), 267–300.

Rissoli, R. Z., Abdalla, F. C., Costa, M. J., Rantin, F. T., McKenzie, D. J., and Kalinin, A. L. (2016). Effects of glyphosate and the glyphosate based herbicides Roundup Original® and Roundup Transorb® on respiratory morphophysiology of bullfrog tadpoles. *Chemosphere* 156, 37–44. doi: 10.1016/j.chemosphere.2016.04.083

Rossberg (2015a). *Use of POEA Free Glyphosate-Based Formulations in German Agriculture.* Personal communication, Kleinmachnow.

Rossberg (2015b). *Use of Glyphosate in Germany by Professional Users Apart from Agriculture for Year 2012.* Personal communication, Kleinmachnow.

Soloneski, S., Ruiz de Arcaute, C., and Larramendy, M. L. (2016). Genotoxic effect of a binary mixture of dicamba-and glyphosate-based commercial herbicide formulations on *Rhinella arenarum* (Hensel, 1867) (Anura, Bufonidae) late-stage larvae. *Environ. Sci. Pollut. Res.* 23, 17811–17821.

Steinmann, H. H., Dickeduisberg, M., and Theuvsen, L. (2012). Uses and benefits of glyphosate to German arable farming. *Crop Protec.* 42. 164–169. doi: 10.1016/j.cropro.2012.06.015

Steinmann, H. H., and Dobers, E. S. (2013). Spatio-temporal analysis of crop rotations and crop sequence patterns in Northern Germany: potential implications on plant health and crop protection. *J. Plant Dis. Prot.* 120, 85–94. doi: 10.1007/BF03356458

Stuart, S. N., Hoffmann, M., Chanson, J. S., Cox, N. A., Berridge, R. J., Ramani, P., et al. (2008). *Threatened Amphibians of the World*. Barcelona: Lynx Editions.

Vincent, K., and Davidson, C. (2015). The toxicity of glyphosate alone and glyphosate–surfactant mixtures to western toad (*Anaxyrus boreas*) tadpoles. *Environ. Toxicol. Chem.* 34, 2791–2795. doi: 10.1002/etc.3118

Wagner, N., and Lötters, S. (2013). *Possible Correlation of the Worldwide Amphibian Decline and the Increasing Use of Glyphosate in the Agrarian Industry. – BfN-Skripten 343*. Bonn: Bundesamt für Naturschutz.

Wagner, N., Reichenbecher, W., Teichmann, H., Tappeser, B., and Lötters, S. (2013). Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environ. Toxicol. Chem.* 32, 1688–1700. doi: 10.1002/etc.2268

Weedscience (2015). *International Survey of Herbicide Resistant Weeds.* Available online at: http://www.weedscience.org/summary/home.asp (Accessed Jan 26, 2015)

Xu, Q., and Oldham, R. S. (1997). Lethal and sublethal effects of nitrogen fertilizer ammonium nitrate on Common toad (*Bufo bufo*) tadpoles. *Arch. Environ. Contam. Toxicol.* 32, 298–303.

Yadav, S. S., Giri, S., Singha, U., Boro, F., and Giri, A. (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (*Euflictis cyanophlyctis*) in the presence and absence of predator stress. *Aquat. Toxicol.* 132-133, 1–8. doi: 10.1016/j.aquatox.2013.01.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AG and handling editor declared their shared affiliation.

Copyright © 2018 Berger, Graef, Pallut, Hoffmann, Brühl and Wagner. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 24 August 2016
doi: 10.3389/fenvs.2016.00051

Temperature-Dependence of Glyphosate-Based Herbicide's Effects on Egg and Tadpole Growth of Common Toads

*Fabian Baier, Mathias Jedinger, Edith Gruber and Johann G. Zaller**

Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Life Sciences, Vienna, Austria

Glyphosate-based herbicide formulations are broadly used in agriculture, silviculture, horticulture as well as in private gardens all over the world, thus posing the risk of potential contamination of nearby aquatic bodies inhabited by amphibians. Concurrently, climate change can be expected to alter the temperature of amphibian breeding sites. However, while either glyphosate-based herbicides or temperature have been shown to separately affect the development of amphibians, very little is known on possible interactive effects. We studied the impact of herbicide concentrations and temperature on growth and development of eggs and tadpoles of the Common toad (*Bufo bufo* L.). We hypothesized that (i) eggs would be better protected against herbicides than tadpoles because of their jelly coating, (ii) that higher temperatures would reduce potential herbicide effects because of an accelerated growth and a lower sensitivity of larger specimens. We conducted one experiment starting with eggs (Gosner stage, GS 8) and another experiment starting with tadpoles (GS 21–24) using a full factorial design with 5 concentrations of the herbicide formulation Roundup® LB Plus (0.0 mg acid equivalent L^{-1}, 0.5, 1.0, or 1.5 mg a.e. L^{-1} and a pulse treatment with 3 times (egg experiment) or 5 times (tadpole experiment) addition of 0.5 a.e. mg L^{-1} over the course of several weeks) and two temperature levels (15 and 20°C). Contrary to our expectation, our results showed that toad eggs are more sensitive to herbicides than tadpoles leading to an averaged 31% increase in total length, tail length, and body length compared to the herbicide-free control. Tadpole morphology, development, or mortality was not influenced by herbicides. There was no correlation between herbicide concentration and the effect strength on eggs or tadpoles. Higher temperature accelerated growth of both eggs and tadpoles. As one of the first we also observed interactive effects between herbicide concentrations and temperature especially for egg development resulting in more pronounced herbicide effects at lower temperatures than at higher temperatures. This is quite remarkable as ecotoxicologial risk assessment studies are usually conducted at a constant temperature, thereby perhaps not adequately examining non-target effects at natural conditions.

Keywords: agrochemicals, Roundup, climate change, tadpole, pesticide, non-target effects, amphibia, *Bufo bufo*

OPEN ACCESS

Edited by:
Pankaj Kumar Arora, M. J. P. Rohilkhand University, India

Reviewed by:
Astrid Rita Taylor, Swedish University of Agricultural Sciences, Sweden
Fernando José Cebola Lidon, Universidade Nova de Lisboa, Portugal

****Correspondence:***
Johann G. Zaller
johann.zaller@boku.ac.at

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *16 May 2016*
Accepted: *19 July 2016*
Published: *24 August 2016*

Citation:
Baier F, Jedinger M, Gruber E and Zaller JG (2016) Temperature-Dependence of Glyphosate-Based Herbicide's Effects on Egg and Tadpole Growth of Common Toads. Front. Environ. Sci. 4:51. doi: 10.3389/fenvs.2016.00051

INTRODUCTION

Glyphosate-based herbicides are the most often used pesticides worldwide utilized in agriculture, viticulture, horticulture, municipalities, on railroad tracks as well as in private gardens and aquatic environments (Baylis, 2000). Since its introduction in 1974 glyphosate use in the agricultural sector rose 300-fold in 40 years (Benbrook, 2016). The active ingredient glyphosate is taken up by the leaves affecting the shikimate pathway of plants furthermore constraining the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan as a consequence of which the plants die (Steinrücken and Amrhein, 1980; Franz et al., 1997). Because glyphosates' mode of action is suggested to affect only enzymes present in plants and some microorganisms (Duke et al., 2012), potential effects on animals are believed to be small (Giesy et al., 2000). However, in recent years studies reporting detrimental side-effects on amphibians exposed to glyphosate-based herbicide formulations are increasing (e.g., Relyea, 2005; Jones et al., 2010; Williams and Semlitsch, 2010; Berger et al., 2013; Wagner et al., 2013). Moreover, not declared surfactants (e.g., polyethoxylated tallow amine) in these formulations are another crucial point to consider as they might be more toxic than the active ingredient itself (Moore et al., 2012; Cuhra et al., 2016; Mullin et al., 2016).

Globally, 32% of amphibian species are identified as threatened and 44% are affected by population declines mainly called forth by climate change, environmental pollution and land use changes (IUCN, 2004; Stuart et al., 2004). Amphibians are considered good bio-indicators for several reasons (Chovanec and Grillitsch, 1994): First, they live in a complex environment, spending their juvenile stadium in water and their life after metamorphosis in terrestrial ecosystems with an action radius of a couple of kilometers. Second, their skin is highly permeable and very sensitive against environmental pollutants (Quaranta et al., 2009). Third, amphibian larvae filter water or rasp surfaces for food (algae, dead organic matter etc.). Pollutants accumulating on and in those substances can lead to a contamination of tadpoles even if pollutant levels in the water are below the level of detection.

Studies conducted with North-American or Australian amphibian species have shown that glyphosate-based herbicides can indeed affect their growth and mortality (Mann and Bidwell, 1999; Relyea, 2004, 2012; Fuentes et al., 2011; Moore et al., 2012; Lanctôt et al., 2014). However, to what extent European amphibian species are affected by herbicides has only rarely been investigated (Brühl et al., 2013; Wagner et al., 2014; Ujszegi et al., 2015). Another, even less investigated aspect is, whether a climate-change induced change in temperature will alter pesticide effects on non-target organisms (Broomhall, 2004; IPCC, 2013). It has been suggested that climate change itself does not affect amphibians, but rather will act in combination with biotic and abiotic factors increasing their effects (López-Alcaide and Macip-Ríos, 2011). Asian amphibian species have shown temperature-dependent toxicity patterns with the pesticide methomyl (Lau et al., 2015). However, Lötters et al. (2014) state that a phenological shift of certain amphibian species toward earlier reproduction due to climate change might expose them less to glyphosate-based herbicides compared to those which do not show a shift in reproduction time. In Europe, the Common toad (*B. bufo*) is the most widespread amphibian species, living in habitats from lowland up to mountainous areas over 2000 m above sea-level (Cabela et al., 2001; Arnold and Ovenden, 2002). Spawning ponds used by Common toads are often located in agriculturally used landscapes, therefore prone to contamination by agrochemicals applied on the nearby fields.

The current experiment focused on the aquatic phase of Common toad development. There are several ways how herbicide formulations can pollute aquatic environments, including, direct overspraying of rivulets, puddles and small ponds, water run-off through heavy rainfall and erosion of herbicide contaminated soil by wind and water (Solomon and Thompson, 2003; Vereecken, 2005; Sasal et al., 2015). Although its molecular properties and high sorption capacity binds glyphosate tightly to soil and organic particles (Hance, 1976), several reports showed that glyphosate and its degradation products (e.g., aminomethylphosphonic acid, AMPA) are leached out of soils (Zaller et al., 2014) and are found in groundwater, rivers, and ponds and that concentration levels can be high (Scribner et al., 2002; Stadlbauer and Fank, 2005; Peruzzo et al., 2008; Battaglin et al., 2009). Furthermore, climate change is thought to increase pesticide application in Europe (Kattwinkel et al., 2011).

The current study was designed to examine whether (i) different concentrations of a glyphosate-based herbicide have different effects on the development of either eggs or tadpoles of the Common toad and whether (ii) water temperature influences potential herbicide effects on eggs or tadpoles. Generally, we expected that higher herbicide concentrations will be more harmful than lower concentrations and that tadpoles would be more susceptible than eggs because the latter are protected with a gelatinous substance which protects them from environmental factors (Mutschmann, 2010). Because of a faster development of amphibians at higher temperatures, we expected herbicide effects to be less pronounced at higher than at lower water temperature.

MATERIALS AND METHODS

Eggs and tadpoles of *B. bufo* were collected in March and April 2014 from a small pond located in the outskirts of Vienna, Austria (48.240948 N, 16.275672 E). The pond is surrounded by a beech forest and a meadow; to the best of our knowledge, no herbicide application occurred in the vicinity of the pond in the last couple of years. *Bufo* spawn strings were hand-collected from different randomly selected clutches, transferred into plastic containers filled with pond water and stored for 2 days in a climate chamber at 10°C. Egg and tadpole development was determined according to Gosner (1960), who used morphological features to classify amphibian eggs and tadpoles starting with egg fertilization (Gosner stage, GS 1) and ending with the tail resorbtion of the fully metamorphosed tadpole (GS 46). Spawns were sampled with permission of the pond owner (City of Vienna, permission

no. MA49-808754/2014/3) and of the Vienna Environmental Protection Agency (permission no. MA22-736519/2013).

Experimental Setup

We conducted two experiments between end of March and May 2014 in the laboratory of the Institute of Zoology, University of Natural Resources and Life Sciences, Vienna, Austria. One experiment started with *B. bufo* eggs (GS 8 "egg experiment") the other one started with *B. bufo* tadpoles (GS 22, "tadpole experiment"; more details below). Larvae at GS 8 are egg shaped embryos characterized by midcleavage, larvae at GS 22 are tadpole shaped hatchlings characterized by transparent tail fins and fin circulation. In both experiments we studied the effects of herbicide concentration (5 levels) and water temperature (2 levels) and their interactions.

Experimental units were transparent polypropylene tubs (length: 28 cm, width: 19 cm, height: 14 cm; type SAMLA, IKEA, Leiden, The Netherlands) filled with 4 L of coal-filtered (EHEIM classic 250; Deizisau, Germany) tap water. To establish the herbicide treatment we used a liquid formulation of Roundup® LB Plus (Monsanto Co., St. Louis, Missouri, USA) containing the active ingredient glyphosate (360 g L^{-1} glyphosate, 486 g L^{-1} Isopropylamine salt). The herbicide was purchased in a garden shop in a 140 ml bottle (Diwoky GmbH, Vienna, Austria). In Austria, Roundup® LB Plus is registered for use in agriculture, viticulture, forestry, horticulture, municipalities, and private gardens. Although application of this herbicide near water bodies is prohibited, we observed that glyphosate-based herbicides are nevertheless applied near streams for instance to control neophytes. In our experiments we studied effects of the commercially available formulation of the herbicide rather than the pure active ingredient glyphosate in order to assess the real-world situation for amphibians. In the tubs containing the toad eggs we established five different herbicide concentrations: (a) a control treatment containing no herbicide (0.0 mg a.e., acid equivalent L^{-1}), (b) three herbicide treatments where the herbicide was added once, at the beginning of the experiment at 0.5 mg a.e. L^{-1}, 1.0 mg a.e. L^{-1}, or 1.5 mg a.e. L^{-1} and c) a pulse treatment with additions of 0.5 a.e. mg L^{-1} on day 0, 3, and 4 after the start when studying egg development (total 1.5 mg a.e. L^{-1}) or 0.5 mg a.e. L^{-1} pulses on day 0, 4, 8, 12, 17 when studying tadpoles (total 2.5 mg a.e. L^{-1}). These glyphosate concentrations in water are within the range of concentrations used in other studies (Relyea, 2005; Bernal et al., 2009; Relyea and Jones, 2009; Jones et al., 2010) and are reported in studies about pesticide pollution in water bodies within agricultural landscapes (Wagner et al., 2013, 2014).

Temperature treatments were established in two climate chambers (Vötsch Type VPL 400, Weiss Umwelttechnik GesmbH, Vienna, Austria), one maintained at 15°C the other one at 20°C. Under each temperature treatment 5 replicates of the 5 herbicide treatments were established making in total 25 tubs per temperature treatment. The air temperature within the climate chambers was monitored with two data loggers (tiny tag, Gemini Data Loggers, West Sussex, United Kingdom). To monitor water quality in the tubs we measured the dissolved oxygen and temperature at least every third day with an oximeter (WTW, type Oxi 90, Weilheim, Germany). Additionally, we measured the pH-value with a pH-Meter (WTW, type pH-95, Weilheim, Germany).

At the start of each experiment 5 randomly chosen eggs or tadpoles were added into each experimental tub resulting in a total of 250 individuals per experiment (5 individuals × 5 replicates × 5 herbicide concentrations × 2 temperatures). Tadpoles were fed with fish food *ad libitum* (TetraMin, Tetra GmbH, Melle, Germany). The experiments were conducted with a permission for animal testing from the Austrian Federal Ministry of Science, Research and Economy (BMWFW; permission no. BMWFW-66.016/0011-II/3b/2013). In the case tadpoles would get harmed, injured or obviously poisoned an euthanization with MS-222 (Tricaine mesylate; Sandoz, Basel, Switzerland) was foreseen.

Measurements

The experiment on egg development started with eggs at GS 8 (Gosner, 1960) and lasted 22 days. We took images of the tadpoles 22 days after the start of the experiment using a digital single lens reflex camera (Nikon D200 with an Nikon DX objective—APS Nikkor 18–70 mm; Nikon, Tokyo, Japan) in order to non-destructively assess morphological changes in size and shape.

The experiment on tadpole development started with new randomly selected tadpoles (GS 21–24) from the same pond where the eggs were collected. Tadpoles were stored in the climate chamber (10°C) for 2 days in a plastic container filled with pond water. Then 5 tadpoles were randomly selected and distributed among the experimental units that were filled with 4 L coal-filtered tap water (same type of tubs as used in egg experiment). The tadpole experiment ran for 24 days. Treatment effects on tadpole morphology were assessed on images taken by a digital camera at day 24 (for 20°C tadpoles) and day 42 (for 15°C tadpoles).

Egg development was on average assessed every third day and tadpole development every sixth day according to Gosner (1960). Therefore, all tadpoles were collected from each tub, transferred into a petri dish with deionized water and examined under a binocular at 6x magnification (NIKON, type SMZ 645, Leuven, Belgium). To assess tadpole development we took photos of all tadpoles at the end of each experiment. Tadpoles were put in a petri dish (diameter 10 cm) with deionized water and photographs of each tadpole were taken using a digital reflex camera (Nikon D200 with an Nikon DX objective—APS Nikkor 18–70 mm; Nikon, Tokyo, Japan) mounted on a tripod. On these images total length (body + tail length), tail length, body length, and body width were measured on all surviving individuals per experimental unit using the open source image analysis software ImageJ (version 1.48v, National Institute of Health, USA).

Statistical Analyses

All data were tested for homogeneity of variance and normal distribution with the Kolmogorov-Smirnov and the Levene test, respectively. Effects of the herbicide and the temperature treatments and treatment interactions on tadpole morphology, development, and mortality as well as on water pH and water oxygen were tested with two-way analyses of variance

(ANOVAs). Tukey tests were used for multiple comparisons between the different concentrations within each temperature treatment. Statistical results at $P < 0.05$ were considered significant. All statistical tests were performed using the software SPSS (Version 20, IBM©, Armonk, New York, U.S.).

RESULTS

Egg Growth and Development

Herbicide concentration significantly affected body length, tail length, and total length (**Table 1**, **Figure 1**). No significant effects of herbicide concentration on body width, mortality, or development were found (**Table 1**, **Figure 1**). Temperature significantly affected all growth parameters, development and mortality of eggs (**Table 1**, **Figure 1**). On average, body length, body width, tail length and total length of tadpoles at 20°C was increased by 63, 57, 86, and 76% compared to 15°C. Temperature accelerated the development, 22 days after the first herbicide application, the development stage at 20°C was GS 36 while tadpoles at 15°C had only reached GS 28; herbicide concentrations had no significant effect on egg development (**Table 1**, **Figure 3A**). Significant herbicide × temperature interactions for body length, body width, tail length and total length resulted in an increased body length of herbicide treated tadpoles by 26%, body width by 19%, tail length by 35%, and total length by 31% at 15°C compared to the no-herbicide control. In contrast, at 20°C body length, tail length, and total length of herbicide treated tadpoles increased by about 3% and body width was decreased by 2% compared to no-herbicide control. In total, 12.4% (31 out of 250 individuals) of the eggs/tadpoles died or had to be euthanized during the course of the experiment. Mortality was significantly affected by temperature but not by herbicide concentrations or temperature-herbicide, with a total of 18.4% (23 ind.) dead eggs/tadpoles at 15°C compared to 6.4% (8 ind.) dead eggs/tadpoles at 20°C averaged across all herbicide concentrations (**Table 1**, **Figure 4**). Water pH was significantly influenced by herbicide concentration and temperature. Across herbicide concentrations pH values measured were 8.31 ± 0.02 vs. 8.14 ± 0.07 at 15 vs. 20°C, respectively; water oxygen at 15°C was 10.07 ± 0.11 mg L^{-1}, vs. 8.85 ± 0.68 mg L^{-1} at 20°C, respectively (**Tables 1**, **2**). No significant influence of herbicide concentration on water oxygen and herbicide by temperature interaction on water oxygen and pH were found (**Tables 1**, **2**).

Tadpole Growth and Development

Herbicide concentrations had no effect on tadpole growth, development or mortality (**Table 3**, **Figures 2**, **3**). However, temperature highly significantly affected tadpole growth and development but not mortality (**Table 3**, **Figure 2**). Body length, body width, tail length, and total length of tadpoles at 20°C was on average increased by 12, 8, 22, and 18%, respectively, compared to 15°C. Only for tadpole development a significant interaction of herbicide concentration and temperature was found: larvae at 15°C reached on average GS 31, whereas at 20°C an averaged GS 39 was reached 24 days after the first herbicide application (**Table 3**, **Figure 3B**). In total, 14% (35 out of 250) of the tadpoles died or had to be euthanized during this experiment, however neither herbicide concentration nor temperature affected tadpole mortality (**Table 3**, **Figure 4**). During the tadpole experiment, water pH was significantly influenced by herbicide concentration and temperature, whereas water oxygen was significantly influenced by temperature only (**Tables 2**, **3**). Across herbicide concentrations, water pH was 8.57 ± 0.08 vs. 8.41 ± 0.16 at 15 vs. 20°C, water oxygen was 12.21 ± 0.64 mg L^{-1} vs. 10.67 ± 1.26 mg L^{-1} at 15 vs. 20°C, respectively. No significant interaction of herbicide concentration and temperature on water oxygen and pH was observed (**Table 3**).

DISCUSSION

To the best of our knowledge, this study shows for the first time that the globally most widely used herbicide Roundup® affects the growth and development of aquatic stages of Common toads. We expected that egg growth and development would be less sensitive to herbicide concentrations than tadpoles due to the protective jelly coating of the eggs. Indeed, other studies showed that jelly coatings protected embryos of North American amphibian species against endosulfan or tebufenozide pesticides (Berrill et al., 1998; Pauli et al., 1999). A decreased hatching success was also observed for eggs of a European frog species (*Rana arvalis)* that were exposed to the insecticide α-cypermethrin (Greulich and Pflugmacher, 2003); however, no comparison to tadpole development was made in that study. In contrast, in our experiment eggs were actually more sensitive to herbicide concentrations than tadpoles. Since no other study looked into effects of glyphosate-based herbicides on Common toad eggs it remains to be investigated if toad eggs are either especially sensitive or if glyphosate-based herbicides are especially harmful to egg stages of amphibians. Overall, a potential contamination by glyphosate-based herbicides of toad spawning ponds located in agriculturally used landscapes can be expected both for egg and tadpole stages as timing of pesticide application has been shown to match the life cycle of amphibians (Berger et al., 2013; Brühl et al., 2013). Clearly, more studies including more amphibian species are needed to estimate whether this effect is species-specific or applicable more widely.

Our experiment showed a significant increase in body length, tail length, and total length at 15°C after herbicide application which we interpret as a consequence of a further nutrient addition by the herbicide. In contrast to our results, Lanctôt et al. (2014) found that snout to vent length of tadpoles decreased by 11.3 and 5.8% when exposed to two different glyphosate-based herbicide formulations, either two pulses of 0.21 mg a.e. L^{-1} Roundup WeatherMax® or 2.89 mg a.e. L^{-1} Vision®, respectively. However, comparisons with our results have to be made with caution as (i) different glyphosate-based herbicide formulations were used containing different adjuvants with potentially ecotoxicological side-effects (Mullin et al., 2016), (ii) different amphibian species (*Rana sylvaticus*) at a different temperature (21.4°C) have been investigated, and (iii) even the active ingredient glyphosate is not a single chemical, but rather a family of compounds with different chemical, physical, and

TABLE 1 | Egg experiment–Results of a 2-way ANOVA on the effect of a herbicide treatment (5 levels) and a temperature treatment (2 levels) on growth and mortality of the Common toad as well as on water oxygen and pH.

Variable	Herbicide conc.		Temperature		Herb. × Temp.	
	F	*P*	*F*	*P*	*F*	*P*
Body length (mm)	6.708	**<0.001**	866.877	**<0.001**	2.883	**0.035**
Body width (mm)	1.659	0.179	416.159	**<0.001**	2.769	**0.040**
Tail length (mm)	5.971	**<0.001**	955.315	**<0.001**	3.522	**0.015**
Total length (mm)	6.724	**<0.001**	1000.230	**<0.001**	3.466	**0.016**
Development (Gosner stage)	0.003	1.000	71.525	**<0.001**	0.016	0.999
Mortality (%)	0.171	0.952	6.429	**0.015**	1.714	0.166
Water oxygen (mg L^{-1})	2.137	0.094	85.706	**<0.001**	1.025	0.406
Water pH	2.617	**0.049**	138.616	**<0.001**	0.650	0.630

Measurements were taken 21 days after the start of the experiment. Significant effects in bold.

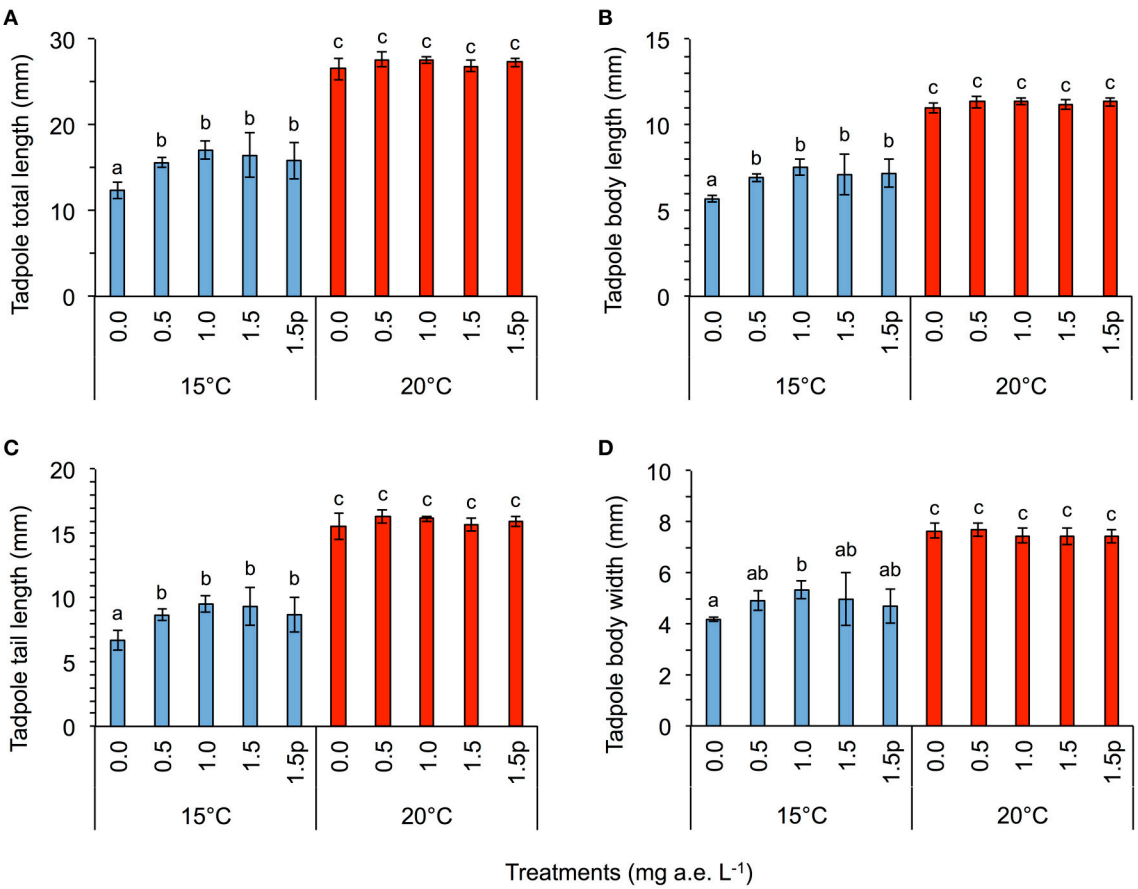


FIGURE 1 | Egg experiment-Average tadpole total length (A), body length (B), tail length (C), and body width (D) in response to five herbicide concentrations (0.0, 0.5, 1.0, 1.5 a.e. mg L^{-1} and a 1.5p a.e. mg L^{-1} pulse treatment) at two temperatures (15 vs. 20°C) at the end of the experiment. Means ± *SD*; $n = 5$. Different letters indicate significant differences between means (Tukey's HSD, $p < 0.05$).

toxicological properties making comparisons between different glyphosate-based formulations even more tricky (Cuhra et al., 2016).

Another hypothesis of our study was that higher herbicide concentrations would show more pronounced growth effects than lower concentrations. Based on the egg and tadpole experiment we can reject this hypothesis as no correlation between herbicide concentration and the strength of the effect on growth, development, or mortality was observed. Reportedly, glyphosate has a half-life of 7–14 days in water (Franz et al.,

TABLE 2 | Water oxygen and pH in the egg and tadpole experiment investigating the effects of herbicide concentrations and temperature on Common toad growth and development.

	Parameters	Water oxygen (mg L^{-1})		Water pH	
		15°C	20°C	15°C	20°C
HERBICIDE CONC. (mg a.e. L^{-1})					
Eggs	0	9.98 ± 0.12ac	8.31 ± 0.09b	8.30 ± 0.01a	8.09 ± 0.03b
	0.5	10.05 ± 0.05ac	8.81 ± 0.57b	8.31 ± 0.01a	8.13 ± 0.07b
	1	10.09 ± 0.12ac	9.21 ± 0.57ab	8.31 ± 0.02a	8.18 ± 0.05b
	1.5	10.03 ± 0.11ac	8.76 ± 0.46b	8.31 ± 0.02a	8.14 ± 0.06b
	1.5p	10.19 ± 0.08a	9.15 ± 1.12bc	8.34 ± 0.02b?	8.18 ± 0.11b
Tadpoles	0	11.60 ± 0.42ab	9.66 ± 1.31b	8.47 ± 0.06ab	8.24 ± 0.16b
	0.5	12.38 ± 0.92a	10.37 ± 0.71b	8.61 ± 0.10a	8.40 ± 0.10bc
	1	12.24 ± 0.42a	11.14 ± 1.80ab	8.59 ± 0.08ac	8.47 ± 0.19ab
	1.5	12.06 ± 0.30a	11.27 ± 0.93ab	8.55 ± 0.03ac	8.47 ± 0.09ab
	2.5p	12.76 ± 0.49a	10.90 ± 1.02ab	8.61 ± 0.06a	8.45 ± 0.14ab

Different letters after values indicate significant differences within herbicide concentrations (Tukey-Tests, $p < 0.05$). Means ± SD, $n = 5$.

TABLE 3 | Tadpole experiment–Results of a 2-way ANOVA on the effect of a herbicide treatment (5 levels) and a temperature treatment (2 levels) on growth and mortality of the Common toad as well as on water oxygen and pH.

Variable	Herbicide conc.		Temperature		Herb. × Temp.	
	F	*P*	*F*	*P*	*F*	*P*
Body length (mm)	0.453	0.770	28.875	**<0.001**	1.200	0.326
Body width (mm)	0.424	0.790	7.293	**0.010**	1.134	0.355
Tail length (mm)	0.337	0.851	72.507	**<0.001**	0.216	0.928
Total length (mm)	0.370	0.828	57.184	**<0.001**	0.505	0.732
Development (Gosner stage)	2.198	0.088	2295.484	**<0.001**	5.789	**<0.001**
Mortality (%)	0.681	0.609	0.000	1.000	1.232	0.313
Water oxygen (mg L^{-1})	2.592	0.051	33.350	**<0.001**	0.864	0.494
Water pH	4.750	**0.003**	25.794	**<0.001**	0.778	0.546

Measurements were taken when tadpoles reached Gosner stage 36–39. Significant effects in bold.

1997; Giesy et al., 2000). Hence, our finding suggests that even low concentrations affected the growth of toad eggs and tadpoles and that the higher concentrated active ingredient might had already disappeared when the growth measurements were conducted. Again, not-declared adjuvants of glyphosate-based herbicide formulations with a higher toxicity than the glyphosate active ingredient itself (Perkins et al., 2000; Edginton et al., 2004; Howe et al., 2004; Peixoto, 2005; Wagner et al., 2013) or their properties as as endocrine disruptors (Gasnier et al., 2009; Defarge et al., 2016) might have contributed to the observed effects. Observed growth stimulations of toad larvae at low herbicide concentrations could perhaps be due to endocrine disruptors as they generally lack a dose-effect relationship (Vandenberg et al., 2012; Vandenberg, 2014). However, the design of our study precludes a substantiation of this explanation. In any case our findings suggest that agrochemical risk assessments that take into account only pesticide active ingredients without their adjuvants commonly used in their comercial formulations will likely miss important toxicological effects (Mullin et al., 2016).

Water temperature showed a consistent strong stimulating effect for both egg and tadpole growth and development. This was expected and has been shown by other studies (Dmitrieva, 2014; Egea-Serrano and Van Buskirk, 2016). Moreover, also mortality was significantly affected by temperature in the egg experiment, where 21% of herbicide treated larvae died at 15°C but only 3% at 20°C. Relyea (2005) determined the lethal concentration where 50% of the population (LC50) of wood frogs (*Rana sylvaticus*) died to be at 1.32 mg active ingredient (a.i.) L^{-1} but the LC50 value of the American toad (*Bufo americanus*) at 2.52 mg a.i. L^{-1}. We assume that the Common toad would react in a similar way, however, it is difficult to compare these studies as different herbicide formulations and different concentrations were used.

To the best of our knowledge, our study is also the first one investigating combined effects of herbicides and temperature on growth and development of a European amphibian species. We found interactive effects of herbicide and temperature on the growth of toad eggs with herbicide effects only seen at 15°C but not at 20°C. This indicates on one hand that toad egg growth

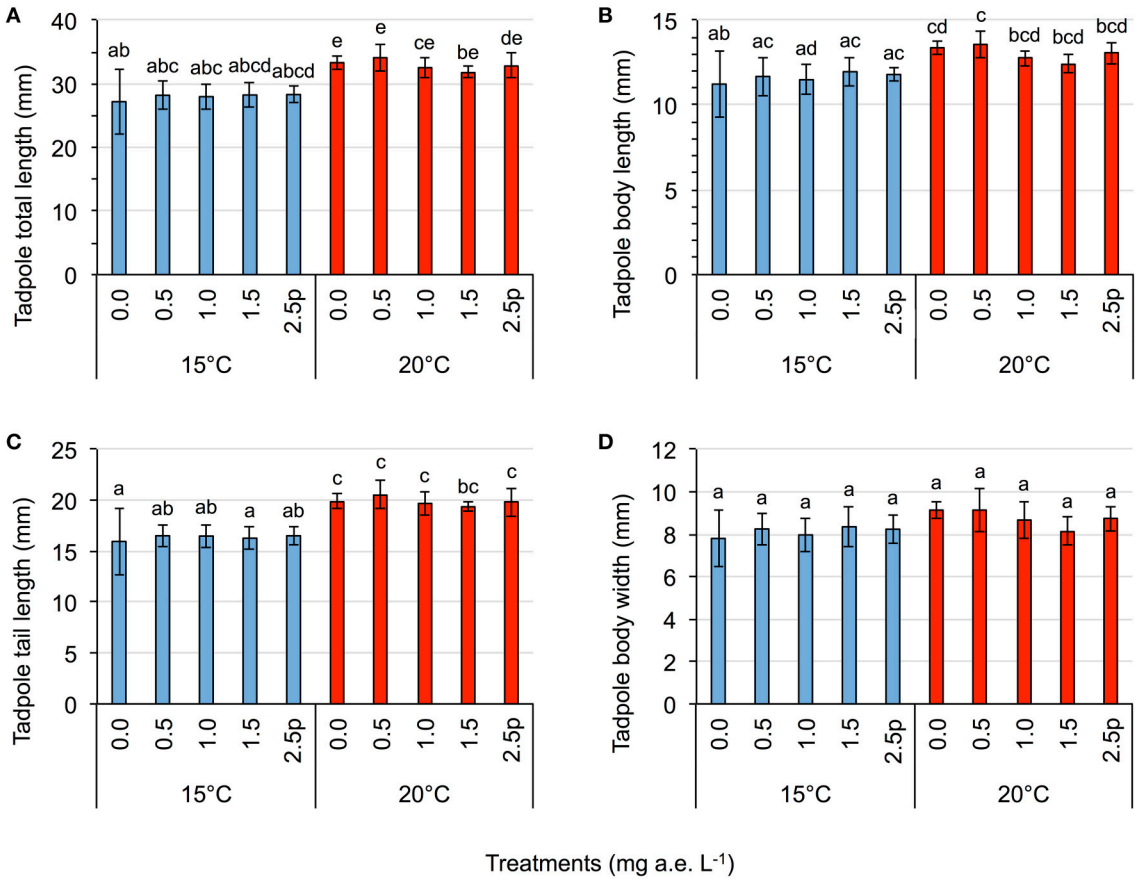


FIGURE 2 | Tadpole experiment–Average tadpole length (A), body length (B), tail length (C), and body width (D) in response to five herbicide concentrations (0.0, 0.5, 1.0, 1.5 a.e. mg L^{-1} and a 2.5p a.e. mg L^{-1} pulse treatment) at two temperatures (15 vs. 20°C) at the end of the experiment. Means ± *SD*, $n = 5$. Different letters indicate significant differences between means (Tukey's HSD, $p < 0.05$).

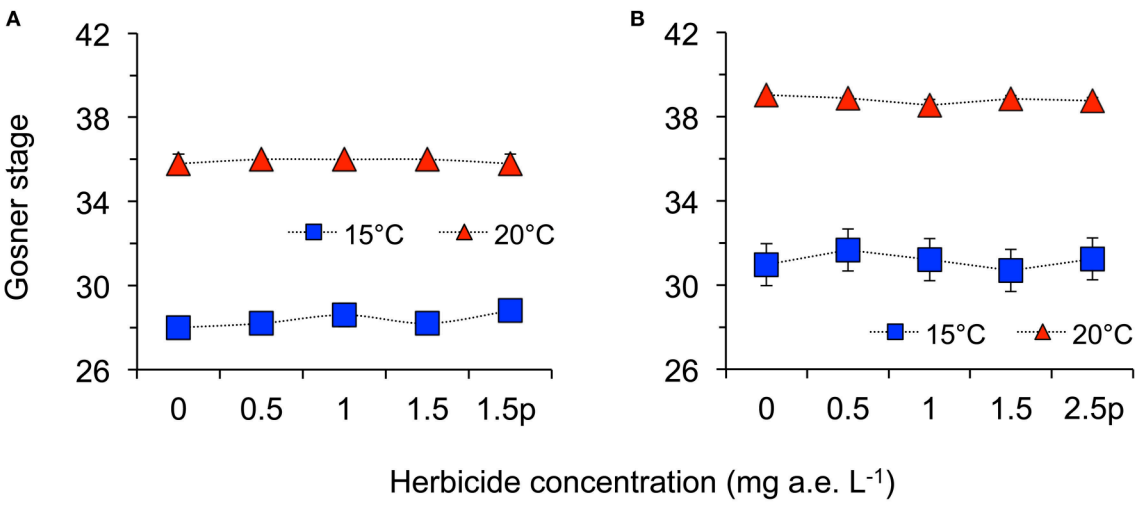


FIGURE 3 | Average development stage according to Gosner (1960) of eggs (A) and tadpoles (B) of the Common toad in response to five herbicide concentrations (0.0, 0.5, 1.0, 1.5 a.e. mg L^{-1} and a 1.5p/2.5p a.e. mg L^{-1} pulse treatment) at two temperatures (15 vs. 20°C) at the end of the experiments; $n = 5$.

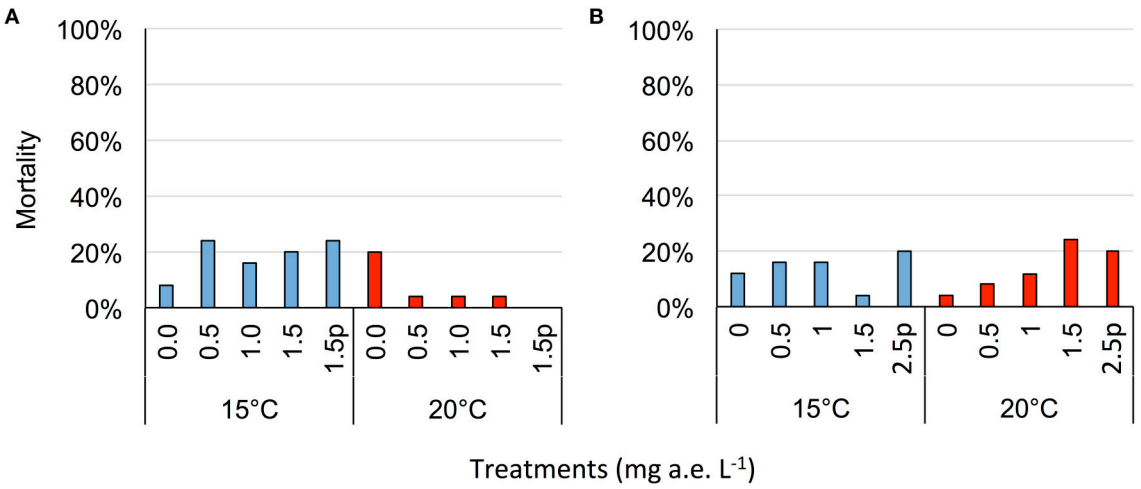


FIGURE 4 | Average mortality (% of total) of eggs (A) and tadpoles (B) of the Common toad in response to five herbicide concentrations (0.0, 0.5, 1.0, 1.5 a.e. mg L^{-1} and a 1.5p/2.5p a.e. mg L^{-1} pulse treatment) at two temperatures (15 vs. 20°C) at the end of the experiment; $n = 5$.

benefited from higher temperatures making them less prone to herbicide concentrations at higher temperature. On the other hand, the tadpole experiment showed that interactive effects of herbicide concentration and temperature were only observed for tadpole development but not for their growth. Tadpole growth also benefited from higher temperatures and was generally less sensitive to herbicide concentration. However, regarding tadpole development the herbicide x temperature interaction indicated a reduced development at 15°C when herbicides were used but no change in development at 20°C.

The current study investigated effects of herbicide concentrations and temperature on Common toad growth and development in a controlled environment. Therefore, an inference of these data to the field situation should be made with caution, as the circumstances in the field are much more complex. For example, predators present in natural sites can trigger morphological changes in amphibian larvae and even enhance these changes in combination with herbicide exposure (Jones et al., 2010). Two other studies found glyphosate-based herbicide formulations (Roundup® and Roundup Original MAX®) being more deadly to larvae of wood frogs (*Rana sylvatica*) and bullfrogs (*Rana catesbeiana*) under a higher stress level (Relyea, 2005; Jones et al., 2011). Although mortality was not influenced by herbicides in our study, it appears that eggs were more stressed under lower temperatures and therefore more susceptible to herbicide concentrations. However, it also has to be noted that most studies, including ours, are rather short term studies and often do not take into account effects of multiple application of various pesticides, which can be even more deleterious to amphibian larvae (Relyea, 2009).

An important implication of our study is that water temperature can alter potential non-target effects of herbicides on amphibian species. This is remarkable as ecotoxicologial risk assessment studies are often conducted at a standard temperature of 20°C which was the temperature showing fewer effects than lower, perhaps more realistic, temperatures.

AUTHOR CONTRIBUTIONS

MJ, EG, and JZ planned and conducted the experiment. FB and MJ wrote the first draft and analyzed the data. All authors reviewed and edited the final manuscript.

ACKNOWLEDGMENTS

We are grateful to Rick Relyea and Carsten Brühl for advice during the planning and carrying out of this experiment. Comments of the reviewers helped to improve an earlier version of this manuscript. This study was partly funded by The Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW, project no. 100977/1).

REFERENCES

Arnold, N., and Ovenden, D. (2002). *A Field Guide to the Reptiles and Amphibians of Britain and Europe*. London: HarperCollins.

Battaglin, W. A., Rice, K. C., Focazio, M. J., Salmons, S., and Barry, R. X. (2009). The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005–2006. *Environ. Monit. Assess.* 155, 281–307. doi: 10.1007/s10661-008-0435-y

Baylis, A. D. (2000). Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Manag. Sci.* 56, 299–308. doi: 10.1002/(SICI)1526-4998(200004)56:4<299::AID-PS144>3.0.CO;2-K

Benbrook, C. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environ. Sci. Eur.* 28, 3. doi: 10.1186/s12302-016-0070-0

Berger, G., Graef, F., and Pfeffer, H. (2013). Glyphosate applications on arable fields considerably coincide with migrating amphibians. *Sci. Rep. (Nature)* 3:2622. doi: 10.1038/srep02622

Bernal, M. H., Solomon, K. R., and Carrasquilla, G. (2009). Toxicity of formulated Glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. *J. Toxicol. Environ. Health A.* 72, 966–973. doi: 10.1080/15287390902929717

Berrill, M., Coulson, D., Mcgillivray, L., and Pauli, B. (1998). Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environ. Toxicol. Chem.* 17, 1738–1744. doi: 10.1002/etc.5620170914

Broomhall, S. D. (2004). Egg temperature modifies predator avoidance and the effects of the insecticide endosulfan on tadpoles of an Australian frog. *J. Appl. Ecol.* 41, 105–113. doi: 10.1111/j.1365-2664.2004.00883.x

Brühl, C. A., Schmidt, T., Pieper, S., and Alscher, A. (2013). Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Sci. Rep.* 3:1135. doi: 10.1038/srep01135

Cabela, A., Grillitsch, H., and Tiedemann, F. (2001). *Atlas zur Verbreitung und Ökologie der Amphibien und Reptilien in Österreich: Auswertung der Herpetofaunistischen Datenbank der Herpetologischen Sammlung des Naturhistorischen Museums in Wien*. Wien: Umweltbundesamt.

Chovanec, A., and Grillitsch, B. (1994). *Amphibien als Bioindikatoren für die Schadstoffbelastung von Kleingewässern*. Wien: Umweltbundesamt.

Cuhra, M., Bøhn, T., and Cuhra, P. (2016). Glyphosate: too much of a good thing? *Front. Environ. Sci.* 4:28. doi: 10.3389/fenvs.2016.00028

Defarge, N., Takaìcs, E., Lozano, V. L., Mesnage, R., Vendômois, J. S., Séralini, G. E., et al. (2016). Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *Int. J. Environ. Res. Public Health* 13:264. doi: 10.3390/ijerph13030264

Dmitrieva, E. V. (2014). Experimental study of the temperature effect on survival and development rates in the early ontogenesis of common toad (*Bufo bufo*). *Moscow Univ. Biol. Sci. Bull.* 69, 71–73. doi: 10.3103/S0096392514020023

Duke, S. O., Lydon, J., Koskinen, W. C., Moorman, T. B., Chaney, R. L., and Hammerschmidt, R. (2012). Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. *J. Agric. Food Chem.* 60, 10375–10397. doi: 10.1021/jf302436u

Edginton, A. N., Sheridan, P. M., Stephenson, G. R., Thompson, D. G., and Boermans, H. J. (2004). Comparative effects of pH and Vision herbicide on two life stages of four anuran amphibian species. *Environ. Toxicol. Chem.* 23, 815–822. doi: 10.1897/03-115

Egea-Serrano, A., and Van Buskirk, J. (2016). Responses to nitrate pollution, warming and density in common frog tadpoles (*Rana temporaria*). *Amphibia-Reptilia* 37, 45–54. doi: 10.1163/15685381-00003029

Franz, J. E., Mao, M. K., and Sikorski, J. A. (1997). *Glyphosate: A Unique Global Herbicide*. Washington, DC: American Chemical Society.

Fuentes, L., Moore, L. J., Rodgers, J. H., Bowerman, W. W., Yarrow, G. K., and Chao, W. Y. (2011). Comparative toxicity of two glyphosate formulations (Original formulation of Roundup® and Roundup Weathermax®) to six North American larval anurans. *Environ. Toxicol. Chem.* 30, 2756–2761. doi: 10.1002/etc.670

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.-C., and Séralini, G.-E. (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology* 262, 184–191. doi: 10.1016/j.tox.2009.06.006

Giesy, J. P., Dobson, S., and Solomon, K. R. (2000). Ecotoxicological risk assessment for Roundup® Herbicide. *Rev. Environ. Contam. Toxicol.* 167, 35–120. doi: 10.1007/978-1-4612-1156-3_2

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183–190.

Greulich, K., and Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquat. Toxicol.* 65, 329–336. doi: 10.1016/S0166-445X(03)00153-X

Hance, R. J. (1976). Adsorption of glyphosate by soils. *Pestic. Sci.* 7, 363–366. doi: 10.1002/ps.2780070407

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., and Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environ. Toxicol. Chem.* 23, 1928–1938. doi: 10.1897/03-71

IUCN (2004). *IUCN Red List of Threatened Species: A Global Species Assessment*. Gland; Cambridge: IUCN.

IPCC (2013). *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK; New York, NY: Cambridge University Press

Jones, D. K., Hammond, J. I., and Relyea, R. A. (2010). Roundup and amphibians: the importance of concentration, application time, and stratification. *Environ. Toxicol. Chem.* 29, 2016–2025. doi: 10.1002/etc.240

Jones, D. K., Hammond, J. I., and Relyea, R. A. (2011). Competitive stress can make the herbicide Roundup more deadly to larval amphibians. *Environ. Toxicol. Chem.* 30, 446–454. doi: 10.1002/etc.384

Kattwinkel, M., Kühne, J. V., Foit, K., and Lies, M. (2011). Climate change, agricultural insecticide exposure, and risk for freshwater communities. *Ecol. Appl.* 21, 2068–2031. doi: 10.1890/10-1993.1

Lanctôt, C., Navarro-Martín, L., Robertson, C., Park, B., Jackman, P., Pauli, B. D., et al. (2014). Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions. *Aquat. Toxicol.* 154, 291–303. doi: 10.1016/j.aquatox.2014.05.025

Lau, E. T., Karraker, N. E., and Leung, K. M. (2015). Temperature-dependent acute toxicity of methomyl pesticide on larvae of three Asian amphibian species. *Environ. Toxicol. Chem.* 34, 2322–2327. doi: 10.1002/etc.3061

López-Alcaide, S., and Macip-Ríos, R. (2011). "Effects of climate change in amphibians and reptiles," in *Biodiversity Loss in a Changing Planet,* ed O. Grillo (Rijeka: InTech), 163–184.

Lötters, S., Filz, K., Wagner, N., Schmidt, B., Emmerling, C., and Veith, M. (2014). Hypothesizing if responses to climate change affect herbicide exposure risk for amphibians. *Environ. Sci. Eur.* 26, 1–5. doi: 10.1186/s12302-014-0031-4

Mann, R. M., and Bidwell, J. R. (1999). The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Arch. Environ. Contam. Toxicol.* 36, 193–199. doi: 10.1007/s002449900460

Moore, L. J., Fuentes, L., Rodgers, J. H., Bowerman, W. W., Yarrow, G. K., Chao, W. Y., et al. (2012). Relative toxicity of the components of the original formulation of Roundup® to five North American anurans. *Ecotoxicol. Environ. Saf.* 78, 128–133. doi: 10.1016/j.ecoenv.2011.11.025

Mullin, C. A., Fine, J. D., Reynolds, R. D., and Frazier, M. T. (2016). Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. *Front. Public Health* 4:92. doi: 10.3389/fpubh.2016.00092

Mutschmann, F. (2010). *Erkrankungen der Amphibien.* Stuttgart: Enke Verlag.

Pauli, B. D., Coulson, D. R., and Berrill, M. (1999). Sensitivity of amphibian embryos and tadpoles to Mimic® 240 LV insecticide following single or double exposures. *Environ. Toxicol. Chem.* 18, 2538–2544. doi: 10.1002/etc.5620181122

Peixoto, F. (2005). Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere* 61, 1115–1122. doi: 10.1016/j.chemosphere.2005.03.044

Perkins, P. J., Boermans, H. J., and Stephenson, G. R. (2000). Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay-*Xenopus*. *Environ. Toxicol. Chem.* 19, 940–945. doi: 10.1002/etc.5620190422

Peruzzo, P. J., Porta, A. A., and Ronco, A. E. (2008). Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environ. Pollut.* 156, 61–66. doi: 10.1016/j.envpol.2008.01.015

Quaranta, A., Bellantuono, V., Cassano, G., and Lippe, C. (2009). Why amphibians are more sensitive than mammals to Xenobiotics. *PLoS ONE* 4:e7699. doi: 10.1371/journal.pone.0007699

Relyea, R. A. (2004). Growth and survival of five amphibian species exposed to combinations of pesticides. *Environ. Toxicol. Chem.* 23, 1737–1742. doi: 10.1897/03-493

Relyea, R. A. (2005). The lethal impacts of roundup and predatory stress on six species of North American tadpoles. *Arch. Environ. Contam. Toxicol.* 48, 351–357. doi: 10.1007/s00244-004-0086-0

Relyea, R. A. (2009). A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. *Oecologia* 159, 363–376. doi: 10.1007/s00442-008-1213-9

Relyea, R. A. (2012). New effects of Roundup on amphibians: predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecol. Appl.* 22, 634–647. doi: 10.1890/11-0189.1

Relyea, R. A., and Jones, D. K. (2009). The toxicity of roundup original max® to 13 species of larval amphibians. *Environ. Toxicol. Chem.* 28, 2004–2008. doi: 10.1897/09-021.1

Sasal, M. C., Demonte, L., Cislaghi, A., Gabioud, E. A., Oszust, J. D., Wilson, M. G., et al. (2015). Glyphosate loss by runoff and its relationship with phosphorus fertilization. *J. Agric. Food Chem.* 63, 4444–4448. doi: 10.1021/jf505533r

Scribner, E. A., Battaglin, W. A., Dietze, J. E., and Thurman, E. M. (2002). *Reconnaissance Data for Glyphosate, Other Selected Herbicides, Their Degradation Products, and Antibiotics in 51 Streams in Nine Midwestern States, 2002*. U.S. Geological Survey Open-File Report 03–217.

Solomon, K. R., and Thompson, D. (2003). Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. *J. Toxicol. Environ. Health Part B Crit. Rev.* 6, 289–324. doi: 10.1080/10937400306468

Stadlbauer, H., and Fank, J. (2005). *Sickerwasserversuche an der Forschungsstation Wagna zur Untersuchung der Verlagerung des Herbizids Glyphosate in der Ungesättigten Bodenzone*. Report Umweltbundesamt. Available online at: http://www.literature.at/alo?objid=18684

Steinrücken, H. C., and Amrhein, N. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochem. Biophys. Res. Commun.* 94, 1207–1212. doi: 10.1016/0006-291X(80)90547-1

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Ujszegi, J., Gál, Z., Mikó, Z., and Hettyey, A. (2015). No observable effect of a glyphosate-based herbicide on two top predators of temporal water bodies. *Environ. Toxicol. Chem.* 34, 307–313. doi: 10.1002/etc.2798

Vandenberg, L. N. (2014). Low-dose effects of hormones and endocrine disruptors. *Vitam. Horm.* 94, 129–165. doi: 10.1016/b978-0-12-800095-3.00005-5

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., and Lee, D. H. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr. Rev.* 33, 378–455. doi: 10.1210/er.2011-1050

Vereecken, H. (2005). Mobility and leaching of glyphosate: a review. *Pest Manag. Sci.* 61, 1139–1151. doi: 10.1002/ps.1122

Wagner, N., Reichenbecher, W., Teichmann, H., Tappeser, B., and Lötters, S. (2013). Questions concerning the potential impact of glyphosate-based herbicides on amphibians. *Environ. Toxicol. Chem.* 32, 1688–1700. doi: 10.1002/etc.2268

Wagner, N., Rödder, D., Brühl, C. A., Veith, M., Lenhardt, P. P., and Lötters, S. (2014). Evaluating the risk of pesticide exposure for amphibian species listed in Annex II of the European Union Habitats Directive. *Biol. Conserv.* 176, 64–70. doi: 10.1016/j.biocon.2014.05.014

Williams, B. K., and Semlitsch, R. D. (2010). Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. *Arch. Environ. Contam. Toxicol.* 58, 819–827. doi: 10.1007/s00244-009-9390-z

Zaller, J. G., Heigl, F., Ruess, L., and Grabmaier, A. (2014). Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Sci. Rep.* 4:5634. doi: 10.1038/srep05634

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Baier, Jedinger, Gruber and Zaller. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

REVIEW
published: 14 August 2017
doi: 10.3389/fenvs.2017.00050

Temperature and Light Modulation of Herbicide Toxicity on Algal and Cyanobacterial Physiology

Marcelo Pedrosa Gomes[1,2]* *and Philippe Juneau*[1]*

[1] *Ecotoxicology of Aquatic Microorganisms Laboratory, Département des Sciences Biologiques – GRIL – TOXEN, Université du Québec à Montréal, Montréal, QC, Canada,* [2] *Laboratório de Fisiologia Vegetal, Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*

HIGHLIGHTS

- We reviewed the interaction between light, temperature and herbicides on algal and cyanobacterial physiology.
- Temperature is the main factor affecting herbicide toxicity to algae and cyanobacteria.
- Changes in light environment may modulate the effects of photosynthesis-targeting herbicides.

Important interactions between climatic parameters and herbicide toxicity have been discussed in the literature. As climate changes are expected to influence the growth conditions of aquatic photosynthetic organisms over the next century by modifying the physicochemical parameters of the environment (such as temperature and incident light characteristics), the following questions arise: How will variations in climatic conditions influence herbicide toxicity in algae and cyanobacteria? Are these coupled effects on aquatic photosynthetic organism physiology antagonistic, additive, or synergistic? We discuss here the physiological responses of algae and cyanobacteria to the combined effects of environmental changes (temperature and light) and herbicide exposure. Both temperature and light are proposed to influence herbicide toxicity through acclimation processes that are mainly related to cell size and photosynthesis. Algal and cyanobacterial responses to interactions between light, temperature, and herbicides are species-specific, making it difficult today to establish a single model of how climate changes will affect toxicity of herbicides. Acclimation processes could assure the maintenance of primary production but total biodiversity should decrease in communities exposed to herbicides under changing temperature and light conditions. The inclusion of considerations on the impacts of environmental changes on toxicity of herbicides in water quality guidelines directed toward protecting aquatic life is now urgently needed.

Keywords: environment, toxicity, hazardous, pollutants, algae, cyanobacteria

OPEN ACCESS

Edited by:
Johann G. Zaller,
University of Natural Resources and Life Sciences, Austria

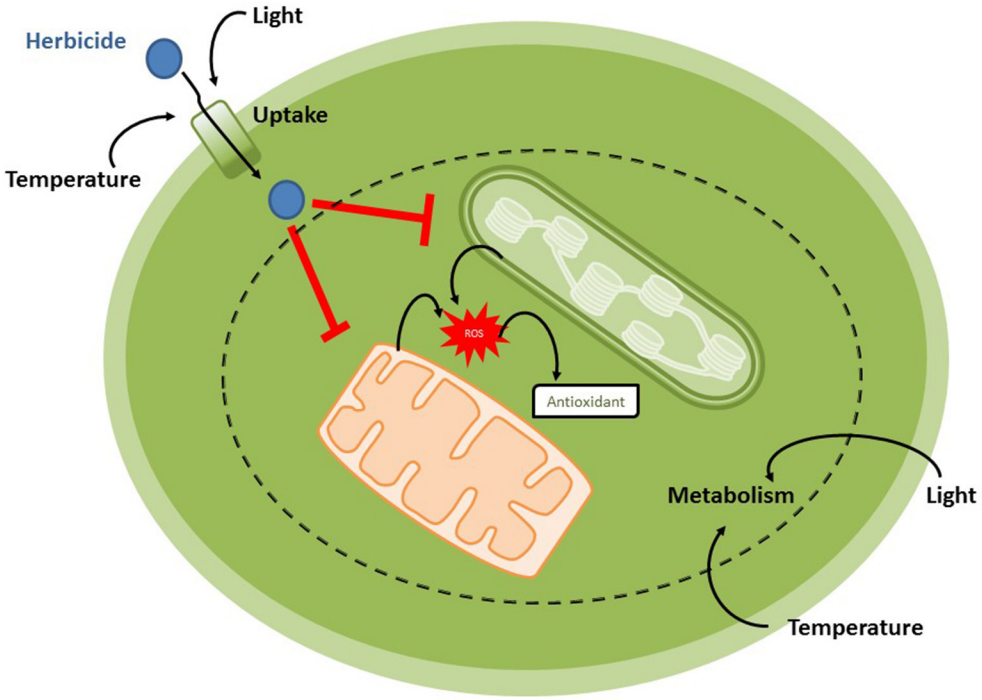
Reviewed by:
Elisabeth Bondar-Kunze,
Wasser Cluster Lunz, Austria
Yohei Shimasaki,
Kyushu University, Japan

***Correspondence:**
Marcelo Pedrosa Gomes
marcelopgom@icb.ufmg.br
Philippe Juneau
juneau.philippe@uqam.ca

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *04 April 2017*
Accepted: *28 July 2017*
Published: *14 August 2017*

Citation:
Gomes MP and Juneau P (2017) Temperature and Light Modulation of Herbicide Toxicity on Algal and Cyanobacterial Physiology. Front. Environ. Sci. 5:50. doi: 10.3389/fenvs.2017.00050



Graphical Abstract | Changes in temperature and light conditions in water systems over the next century will drive physiological responses of algal and cyanobacterial community to herbicides, by antagonistic, additive, or synergic effects with these pollutants.

INTRODUCTION

Climatic changes are expected to influence aquatic photosynthetic organism growth conditions over the next century by modifying physicochemical parameters of the environment such as temperature, precipitation, and incident light characteristics (Finkel et al., 2010). Changes in water temperature, pH, UV-B in the upper water column (due to ozone depletion), and the introduction of pollutants into waterways induce stress on algal and cyanobacterial communities (Finkel et al., 2010), affecting their physiological processes. Due to the central role of aquatic photosynthetic organisms for global primary production, the identification of their physiological responses to these changes is essential to our understanding of how these natural and anthropogenic alterations of the aquatic ecosystems will affect the carbon-climate system at a global scale.

Parameters linked to the photosynthetic activity of plants have been shown to be useful to study responses to stress conditions (Juneau et al., 2007), mainly because photosynthesis is directly associated with growth and primary production. Several studies have examined the effects of climatic factors (such as temperature and irradiance) on photosynthesis of aquatic organisms (Huner et al., 2003; Necchi Jr, 2004). Temperature, for example, has been observed to modulate algal and cyanobacterial photosynthesis by modifying pigment and lipid compositions (and thus membrane fluidity) and enzyme activities (Huner et al., 2003; Necchi Jr, 2004; Chalifour et al., 2014). Although photoacclimation processes allow aquatic organisms to adjust their photosynthetic apparatus in response to changing light environments, prolonged exposure to high light intensities can induce photoinhibition and photodamage (Huner et al., 2003).

The development of intensive agriculture in areas near aquatic ecosystems had led to increased agricultural waste load entering these waterways (Costanzoi and Dennison, 2003). Among the agricultural wastes, nutrient inputs, such as phosphates and nitrates, are known to increase eutrophication of lakes, rivers and coastlines (Costanzoi and Dennison, 2003), and these nutrients are rarely limiting in these ecosystems. In most laboratorial studies on algal physiological responses, media used contain enough nutrients (like in agricultural area aquatic ecosystems) to allow maximum growth rate, which therefore, are not problematic for physiological processes associated to growth and photosynthesis. Therefore, in this review, the effects of agricultural nutrient inputs will not be considered. In contrast, anthropogenic additions of hazardous compounds to waterways can significantly affect photosynthesis of aquatic organisms (DeLorenzo et al., 2001), and agrotoxics (mainly herbicides) heavily used in agricultural and industrial practices are notorious for their effects on algal and cyanobacterial physiology and growth (Bérard et al., 1999; Chalifour and Juneau, 2011). Photosynthesis-targeting herbicides, such as atrazine, simazine and terbuthylazine, act primarily on photosynthetic light reactions and inhibit carbon assimilation (Jursinic and Stemler, 1983). On the other hand, even if photosynthesis and respiration are not the primary target of some herbicides (such as glyphosate), these metabolic pathways may be also

affected by these substances (Qiu et al., 2013; Gomes and Juneau, 2016; Gomes et al., 2016). Although it has been banned or restricted in several countries, PSII inhibitors such as atrazine, simazine and terbuthylazine can be detected in the majority of surface waters of agricultural areas (Comber, 1999). Together with glyphosate, the most used herbicide worldwide, photosynthesis-targeting herbicides are important contaminants of waters, and therefore, the present review focused on the interaction between environmental factors and these herbicides. **Table 1** shows some examples of maximal concentrations of herbicides found in waters around the world. Although light is essential to perform photosynthesis, adequate levels (those that do not induce photoinhibition) are needed otherwise reactive oxygen species (ROS) accumulation and photosynthetic activity decrease will occur (Waring et al., 2010; McGinty et al., 2012). Similarly, temperature needs to be optimal in order to maintain maximal enzyme activity (Huner et al., 2003; Necchi Jr, 2004), including antioxidant enzymes necessary to keep ROS under non-harmful physiological levels. The deleterious effects of some herbicides on photosynthesis have been shown to be associated with cell oxidative bursts due to ROS accumulation (Jursinic and Stemler, 1983; Cuypers et al., 2001; Romero et al., 2011; Gomes et al., 2016) which promotes lipid peroxidation, leading to the destruction of cell membrane systems such as, chloroplast thylakoids (Li et al., 2006; Hao et al., 2010). In this context, a physiologically interconnected interpretation of the environmental condition (natural and anthropogenic factors) impacts on aquatic organism's primary production is needed.

The impacts of some herbicides such as the bipyridinium (i.e., diquat and paraquat) and fluometuron are closely linked to climatic factors such as light. Indeed, in presence of light, bipyridinium herbicides inhibit photosynthesis by intercepting electrons from the reducing side of PSI, forming relatively stable free radicals; in the presence of oxygen, the bipyridinium free radical is then oxidized, which leads to the formation of a highly activated oxygen species that promote cellular oxidative bursts (DeLorenzo et al., 2001). Fluometuron, in turn, on the top of its action on PSII electron transport (Wilkinson et al., 2015) inhibits carotene biosynthesis and deprives cells of their photo-oxidative carotene shield—leading to chlorophyll degradation and decreases in photosynthetic rates (DeLorenzo et al., 2001). In the absence of light, however, neither of these herbicides is effective (Sikka and Pramer, 1968; Corbett et al., 1984). Similarly, temperature has been shown to modulate atrazine toxicity in microalgae (Chalifour and Juneau, 2011). These results indicate the existence of important interactions between climatic parameters and the toxicity of herbicides. In this context, the following questions arise: How do variations in climatic conditions influence herbicide toxicity in algae and cyanobacteria? Are their coupled effects on photosynthetic aquatic organism's physiology antagonistic, additive, or synergistic?

We discuss here the physiological responses of algae and cyanobacteria (both phytoplanktonic and periphytic since their physiological processes are similar) to the combined effects of environmental changes (temperature and light) and herbicide exposure. Firstly, physiological processes associated to aquatic photosynthetic organism acclimation to changes in temperature and light conditions are presented. Then, the interactive effects of changes in temperature or light conditions and herbicide toxicity to algae and cyanobacteria are discussed. In addition to contributing to the field of plant physiology, this review provides unique perspectives for better understanding natural aquatic photosynthetic organism community responses to herbicides in the context of climate change.

TABLE 1 | Examples of maximal concentrations of herbicides (PSII-inhibitors and glyphosate) in waters of different regions.

Herbicide	Region	Maximal concentration ($\mu g\ l^{-1}$)	Authors
Atrazine	Europe	25.0	Croll, 1991; Legrand et al., 1991
	Australia	150.0	Graymore et al., 2001
Simazine	Europe	0.25	Carafa et al., 2007
Terbuthylazine	Europe	0.69	Carafa et al., 2007
Diuron	Canada	0.47	Giroux, 2015
Isoproturon	Europe	0.12	Reupert and Ploeger, 1989
Terbutryn	Europe	5.6	Quednow and Püttmann, 2007
Glyphosate	Europe	50.0	Horth and Blackmore, 2009
	Canada	48.8	Giroux, 2015

ACCLIMATION

Acclimation is the process by which organisms can adjust to environmental changes and maintain their performance levels under new environmental conditions. By modifying their physiological responses, aquatic photosynthetic organism communities can deal with different conditions of temperature, light (Davison, 1991), and contaminant exposure. Although acclimation processes may initiate within just few hours (Davison, 1991), acclimation/adaptation times reported for microalgae can vary from hours to many weeks (Davison, 1991; Chalifour and Juneau, 2011; Larras et al., 2013)—an important aspect that must be considered in studies evaluating algal and cyanobacterial acclimation in response to new environmental pressures.

Thermal-Acclimation

Temperature is an important regulating factor for primary production in aquatic environments as it controls fundamental organism functional properties such as photosynthesis, respiration, and nutrient uptake (Staehr and Sand-Jensen, 2006). Photochemical processes are influenced by temperature since membrane fluidity and diffusion of electron carriers are modulated by temperature changes (Los and Murata, 2004). Optimum temperatures for photosynthesis, when carbon assimilation is the highest, are species-specific. In general, however, at low temperatures, the viscosity of the membranes is increased, preventing the transport of associated components

into the membranes, while the reverse is observed at higher temperatures (up to a limit where degradation of the membranes and proteins is observed) (Barber et al., 1984). Moreover, temperature modulates intermolecular collision processes of electron carriers (such as plastoquinone and plastocyanin) that independently of any effects on membrane fluidity *per se*, increases the oxidation-reduction turnover times of these components under low temperatures (Falkowski and Raven, 2013). Low temperatures also induce decreased Rubisco activity (Staehr and Sand-Jensen, 2006). This constraint gradually vanishes, however, as temperature increases across the optimum temperature range, stimulating carbon fixation (Staehr and Sand-Jensen, 2006). The Calvin cycle enzyme activity also represents a point of temperature modulation of plant photochemistry. In this context, the maximum rate of electron transport is lower under low temperatures and accelerated under higher temperatures (Pimentel et al., 2007).

Similarly to Calvin cycle, respiratory processes are less active under lower temperatures and activated at higher temperatures, demonstrating that temperature can also modulate mitochondrial activities (Atkin and Tjoelker, 2003). There is an optimal temperature for photosynthesis; beyond that temperature, the anabolism of carbon skeleton molecules (by respiration, for instance) are greater than their production (by photosynthesis) which leads to decreasing growth rates (and increases in light requirements for survival). Moreover, if temperatures continue to rise, higher membrane fluidity and enhanced protein and enzyme degradation rates (Daniel et al., 1996; Los and Murata, 2004) will decrease aquatic photosynthetic organism performances. It is also important to note that increased metabolism also increases nutrient demands (Rhee and Gothan, 1981). Nutritional status is directly linked to pigment and enzyme concentrations and, as nutrient availability will vary among different aquatic systems and seasons, it can markedly influence metabolic processes in response to temperature changes (Raven and Geider, 1988). Similarly, increased membrane fluidity can result in increased cell permeability to water pollutants, which will also modulate algal and cyanobacterial responses to temperature changes.

Algal and cyanobacterial species have the ability to acclimate to changes in water temperatures and can alter their physiological processes within just a few generations (Vona et al., 2004). At low temperatures, chlorophyll/cell ratio tends to become reduced, while carboxylation activity tends to increase (through increasing Rubisco levels) (Davison, 1991). These alterations lead to the reduction in light absorption capacity to avoid photoinhibition, while increasing carbon assimilation. Thus, aquatic photosynthetic organism adaptations to low temperatures are basically through acclimation to high photosystem II excitation pressure (Maxwell et al., 1995). In contrast, under high temperature conditions, thermal acclimation assures a balance between photosynthesis and respiration, and the temperatures required for optimal photosynthesis rates are often lower than those of respiration (Raven and Geider, 1988). This means that high temperatures will constrain photosynthesis more than respiration, resulting in higher consumption rates of ATP and carbohydrates than production rates (Raven and Geider, 1988), so that mechanisms allowing acclimation of the photosynthetic apparatus will be crucial in determining algal and cyanobacterial survival. Thermal acclimation modulates also antioxidant system activities that protect proteins and membranes from oxidative stress induced, for example, by low temperatures (Suzuki and Mittler, 2006). Changes in lipid composition is also a common response of thermal acclimation (mainly to low temperatures) to adjust membrane fluidity (Falkowski and Raven, 2013), thereby maintaining PSII stability (Hölzl and Dörmann, 2007) while allowing the movement of components in the electron transport chain (Sarcina et al., 2001).

The influence of temperature on algal and cyanobacterial pigment contents, especially on chlorophyll *a* (which is generally used to estimate the biomasses of photosynthetic microorganisms), has also received substantial attention—but inconsistent results have been reported in the literature. Increased chlorophyll content at 10°C compared to 25°C have been observed in the mesophilic species *Scenedesmus obliquus* (Chalifour and Juneau, 2011). Similarly, chlorophyll and carotenoid contents were lower in the green algae *Chlamydomonas reinhardtii* grown under 15°C compared to 25°C (Chalifour et al., 2014). Chen et al. (2010), in turns, observed an increased chlorophyll content in the cyanobacteria *Microcystis aeruginosa* grown at 25°C in relation to 10°C. On the other hand, chlorophyll content was not significantly affected by temperature in the diatom *Navicula pelliculosa* (at 10, 15, and 25°C) (Chalifour and Juneau, 2011) or in the cyanobacteria *Merismopedia tenuissima* and *Oscillatoria sp*. (at 15, 20, 25, and 30°C) (Coles and Jones, 2000). Temperature effects on pigment content may therefore vary depending on the algal and cyanobacterial species, the growth temperature, and light intensity. The unimodal relationship between functional properties and temperature can be altered by acclimation processes (Staehr and Sand-Jensen, 2006). Since aquatic photosynthetic organisms can tolerate relatively large changes in environmental temperatures through the adjustment into their cellular physiology (Staehr and Birkeland, 2006), the effects of global warming on algal and cyanobacterial productivity could be less dramatic than predicted on studies based on models using non-acclimated species (Atkin and Tjoelker, 2003).

Photo-Acclimation

Aquatic organisms are exposed to variable light intensities throughout the growing season due to daily and seasonal annual changes in light availability, or to their position in the water column (Dubinsky and Stambler, 2009). These modifications in light intensity are amplified by the physical properties of the water and by the presence of suspended particles and dissolved chemicals that attenuate light, creating highly variable light environments depending on the habitats (Smith and Mobley, 2008). High light levels during sunny days can be sufficient to induce photoinhibition, while light attenuation by the water column may impose restraints on photosynthetic organisms just a few meters below the surface (Smith and Mobley, 2008). Within

this context, photoacclimation processes aid photosynthetic aquatic organism's survival under changing light conditions.

Morphological traits such as interspecific cell size variations, the formation of colonies, filaments, or cell clusters, and cell motility will affect light use efficiency in phytoplankton (Deblois et al., 2013). Colonial organization is an ecological gain since it provides grazing protection; however, it stablish a condition of lower light availability for cells, once this kind of organization increases density (and thus the sinking rate) at the same time that promotes self-shading (Falkowski and Raven, 2013). Flagella or vacuoles allow phytoplankton to move in the water column and optimize light harvesting (Falkowski and Raven, 2013). Pigment variability (chlorophyll, carotenoids, and phycobiliproteins) is also other source of variation in phytoplankton light harvesting capacity (Falkowski and Raven, 2013). It is also known that the production of ultraviolet-radiation absorbing micosporne-like amino acids (MLAAs) in cyanobacteria varies with the intensity of photosynthetically active radiation (PAR) (Torres et al., 2016). Together, these characters can influence photoacclimation processes and help to explain the different photoacclimation strategies observed among different algal and cyanobacterial species.

Photoacclimation mechanisms tend to equilibrate light energy harvested and the energy used in metabolism in order to maximize adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) production in accordance with cellular activities (Falkowski and Raven, 2013). Some important photoacclimation mechanisms involve modifications in: the sizes of light harvesting complexes (LHC); pigment compositions and concentrations; and the stoichiometry of PSI and PSII, and the components of the electron transport chain (MacIntyre et al., 2002). Changes in LHC size allow phytoplankton to increase or decrease the energy delivered to their photosystems (Behrenfeld et al., 2004). Similarly, modifications of chlorophyll contents modulate photon harvesting—increasing or decreasing the energy processed in the photosynthetic apparatus (Deblois et al., 2013). Additionally, as chlorophyll is an important component of photosynthetic reaction centers (RC), modulation of chlorophyll contents will induce changes in RC stoichiometry (Mauzerall and Greenbaum, 1989). In addition to the protective roles of photoprotective pigments (i.e., carotenoids), changes in their concentrations also help control the amounts of energy directed to the PS during light harvesting by increasing or decreasing light dissipation as heat (Kirilovsky, 2007). The non-photochemical processes were also shown to play a critical role in protection against high light when diatoms are exposed to a combination of environmental factors (Juneau et al., 2015). Finally, changes in PS stoichiometry can increase or decrease PSI to PSII ratios, equilibrating the flow of energy from PSII to PSI and reducing light pressure on the photosynthetic apparatus (Sonoike et al., 2001). Under low light (LL) conditions, phytoplankton adjust their cellular constituents to increase their light absorption efficiency, which involves down-regulation or *de novo* synthesis of photosynthetic (chlorophyll) and photoprotective pigments (carotenoids), PS, Rubisco, and ultrastructural cell alterations (Herzig and Dubinsky, 1992). Under high light (HL) conditions, in turns, photoacclimation allows phytoplankton to avoid photoinhibition and oxidative damage by decreasing their chlorophyll and increasing their carotenoid contents—which are associated with decreases in the numbers of PS and changes in LHC size (MacIntyre et al., 2002).

Photoinhibition and oxidative stress are closely related. ROS are continuously produced during chloroplast activity: the triplet chlorophyll, the electron transport chain, and the oxygen evolution complex are all ROS production sites (Gomes and Garcia, 2013), and the over-excitation of PS by HL induces ROS production in phytoplankton (Herzig and Dubinsky, 1992). Within this context, the activity of antioxidant systems (i.e., antioxidant-enzymes) is another site of modulation of the photoacclimation process (MacIntyre et al., 2002). Little attention, however, has been given to this important physiological aspect that may be intrinsically related to algal and cyanobacterial survival under light changing environments.

Although acclimation processes may assure their survival under environmental changes in temperature and light conditions, algae and cyanobacteria in aquatic systems are also exposed to hazardous compounds, such as herbicides, and their ability to cope with these compounds may be influenced by the physiological responses to changes in temperature and light conditions. In the following section, the interaction between temperature/light herbicides is discussed.

TEMPERATURE AND HERBICIDES IN PHYTOPLANKTON AND PERIPHYTON

General Concern

Anthropogenic disturbances that occurred over the past century have driven relatively rapid changes in environmental temperatures resulting in global warming, affecting both land and aquatic habitats (Knutti et al., 2016). As temperature is a major determinant of the broad-scale distribution of organisms (Haidekker and Hering, 2008), one important factor of biological responses to climate change will be the extent of those temperature changes (Deutsch et al., 2008).

Fluctuations in growth temperatures are known to alter the effects of chemical stressors (Folt et al., 1999; Chalifour and Juneau, 2011), but the implication of these interactive effects has not been yet addressed in standard toxicity test protocols (Folt et al., 1999). The interactions of combined chemical and physical stressors may create effects that are either greater than, or lesser than, those of each stressor alone (Crain et al., 2008). Consequently, it is essential to understand when natural variations in environmental variables modify the toxicity of water pollutants and to incorporate this knowledge into environmental regulation and risk evaluation and mitigation strategies (Chen et al., 2008).

Pollutants and temperature are two environmental factors that can affect algal and cyanobacterial photosynthesis and growth in natural environments. Pollutants often inhibit photosynthesis and growth, which are also affected by changes in temperature. Temperature modulates the enzymatic activities involved in repair processes (including the activation, synthesis, or repair

of damaged molecules) (Sobrino and Neale, 2007) needed to counteract the damages induced by exposure to pollutants. Temperature can also interact additively or synergistically with pollutants, accentuating their detrimental effects on cellular mechanisms (Bicalho et al., 2017).

Numerous studies have demonstrated the increasing frequency of chronic or acute pollution of waterways with high levels of pesticide (especially herbicide) residues (IFEN, 2006; Sullivan et al., 2009; Giroux, 2015). Aquatic environments near agricultural fields receive direct and indirect herbicide inputs that may impact the primary production of algae and cyanobacteria communities (and therefore other trophic levels) (DeLorenzo et al., 2001). In the following sections the interaction between temperature and some herbicides are reviewed.

Case of PSII Inhibitors and Temperature

Atrazine (6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4,diamine) is the most frequently detected herbicide in aquatic systems (Rebich et al., 2004; Giroux, 2015). Atrazine concentrations can exceed the general quality standard for surface and drinking water ($5\,\mu g\ l^{-1}$) (Giroux, 2015). Values ranging from 9 to $25\,\mu g\ l^{-1}$ in Europe (Croll, 1991; Legrand et al., 1991), 87 to $100\,\mu g\ l^{-1}$ in North America (Steinheimer, 1993; Graymore et al., 2001), and up to $150\,\mu g\ l^{-1}$ in Australia (Graymore et al., 2001) have been reported. Although it has been proposed an acute toxicity threshold value of $20\,\mu g$ atrazine l^{-1}, (Diana et al., 2000), under concentrations lower than $20\,\mu g\ l^{-1}$, atrazine was reported to cause sub-lethal effects in various aquatic organisms such as unicellular algae (DeLorenzo et al., 2001). This herbicide interacts with the Q_b-binding site on the D1 protein of PSII, resulting in the inhibition of photosynthetic electron transport with consequent ROS formation (Jursinic and Stemler, 1983). ROS accumulation ultimately induces cellular oxidative bursts that damage pigments and proteins, and the lipids of the photosynthetic apparatus (Gonzáles-Barreiro et al., 2004). Atrazine and temperature are therefore both environmental factors that affect algal and cyanobacterial photochemistry, and their interactive effects have been previously studied (Bérard et al., 1999; Debenest et al., 2010; Chalifour and Juneau, 2011; Larras et al., 2013). Since atrazine is mainly applied when water temperatures are still low (<20°C) in the Northern hemisphere, its interaction with temperature is essential to take into account. The 96-h EC_{50} values for growth rate in the green algal species *Raphidocelis subcapitata* exposed to atrazine ($0–250\,\mu g\ l^{-1}$) were diminished with decreases in temperature (20 to 5°C), indicating a higher toxicity at lower temperature (Baxter et al., 2016). In an *in vivo* experiment, Bérard et al. (1999) observed that the cyanobacteria *Oscillatoria limneltica* was twice as sensitive to atrazine ($10\,\mu g\ l^{-1}$) at low temperature (13°C) compared to higher temperature (20°C). The EC_{50} for *O. limnetica* was $24.2\,\mu g\ l^{-1}$ of atrazine at 13 °C, and $52.3\,\mu g\ l^{-1}$ of atrazine at 20 °C. This temperature-sensitivity response was attributed to the low turnover rates of D1 proteins, the specific target site of this herbicide, at low temperature (Bérard et al., 1999). Since D1 protein turnover is less effective at low temperatures (Ross and Vincent, 1988), low temperatures contribute to the deleterious effects of atrazine on the photosynthetic apparatus by decreasing PSII activity recovery after atrazine binding. It is important to note here that cyanobacteria are more characteristic of aquatic systems at warmer temperatures (Butterwick et al., 2004). Therefore, the increased sensitivity of cyanobacteria species to atrazine under low temperatures could be also accentuated by the sensitivity of these species to low temperatures (Elliott, 2010)—a relevant aspect to be considered in interaction studies.

Chalifour and Juneau (2011) attributed the toxic effects of atrazine on phytoplankton growing at low temperatures to their capacity for cell thermal energy dissipation (non-photochemical energy dissipation processes). They also shown that atrazine ($0.1\ \mu M = 21.56\,\mu g\ l^{-1}$) reduced the electron transport rate (ETR) and kept the plastoquinone pool in an oxidized state (similarly to light limitation effect) in the green algae *S. obliquus* and in the cyanobacteria *M. aeruginosa*. Light limitation of the PSII is known to up-regulate LHCII polypeptide and chlorophyll *a* accumulation (Wilson and Huner, 2000), increasing light absorbing capacity of the cells. Due to the blockage of electron transport by atrazine, increased light absorption will therefore result in increased photo-oxidative damage. Additionally, decreases in ETR due to atrazine exposure decreased xanthophyll-cycle-dependent non-photochemical quenching in the green algal cells (Chalifour and Juneau, 2011) that is dependent on the trans-thylakoid ΔpH (Gilmore, 1997). Decreased non-photochemical quenching (NPQ) was reported for green algae exposed to 0.1 μM atrazine at 10 and 15°C (Chalifour and Juneau, 2011). These authors also noted that a toxic *M. aeruginosa* strain had greater reduction in NPQ at 15°C than at higher temperatures and was more sensitive to atrazine than the non-toxic strain. Thus, we may advance that atrazine-sensitive populations showed reduced abilities to cope with excess energy at low temperatures. On the other hand, these same authors observed increased effective dissipation in active reaction centers (DI_0/RC) in the diatom *N. pelliculosa* at low temperatures (10°C) when exposed to atrazine. This increase in the efficiency of excess energy dissipation was related to the high atrazine tolerance of this diatom (as compared to the other species studied). In their study on *Anabaena circinalis* populations acclimated to increased photon flux densities (PFD - 50, 130, and 230 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ of PAR), Millie et al. (1992) observed that decreasing photosynthetic activity and pigment content (chlorophyll *a* and carotenoid) coincided with increasing PFD. Interestingly, increased sensitivity to simazine (another triazine herbicide that also blocks PSII electron transport) was also seen with increased PFD. These authors concluded that sensitivity to PSII inhibitors was influenced by alterations in the pigment content. High light and low temperature acclimation are similar conditions in terms of light excitation pressure (Maxwell et al., 1995) and, in this context, the acclimatization of photosynthetic aquatic organisms sensitive to atrazine is related to their pigment contents and their thermal energy dissipation abilities. The important role of carotenoids as ROS scavengers and in protecting photosystems from oxidative bursts by quenching the deleterious effects of triplet chlorophyll and singlet oxygen is well-established (Boussiba, 2000). Additionally, since β-carotene is involved in the xanthophyll biosynthetic pathway in algal cells (Bouvier et al., 1996), modulation

of carotenoid contents could directly affect their NPQ ability.

In several countries where atrazine was banned for agricultural usage, triazine (PSII-inhibitors), such as terbuthylazine (TBA) and simazine (SIM), has been used as substitutes, and significant concentrations (from 0.57 to 694.32 ng l^{-1} and 1.45 ng l^{-1} to 25.96 ng l^{-1} for TBA and SIM, respectively) of these herbicides and their byproducts were observed in aquatic environments (Carafa et al., 2007). It was found that concentrations of these herbicides in aquatic environments followed a clear seasonal pattern, with higher concentrations being detected during spring periods (Carafa et al., 2007). Studying the effects of temperature increase (from 15°C to, 25°C) on cellular responses of the marine diatom *Skeletonema marinoi* to environmental to sub-lethal levels (1, 5, 10, 20, and 30 $\mu g\ l^{-1}$) of TBA, Fiori and Pistocchi (2014), observed that this species was quite resistant to TBA exposure at 15°C. However, enhanced temperature increased the algal sensitivity to this herbicide. According to the authors, the low division rates associated with the high nutrient uptake rates at the lowest temperature may contribute with the gradual adaptation of the algal photosynthetic apparatus to the PSII inhibition caused by TBA. Moreover, it is shown that diatoms were more sensitive to triazine herbicides than other species (Bérard et al., 1999; Bérard and Benninghoff, 2001; Chalifour and Juneau, 2011) including harmful flagellate species, specially under rising temperatures. Therefore, the shift in photosynthetic aquatic community composition due to herbicide run off in coastal areas associated with increasing temperatures could led to losses in some essential species for global primary production such as *S. marinoi*.

Other important PSII inhibitor herbicides observed in aquatic systems are diuron, isoproturon and terbutryn, with concentrations up to 0.47 $\mu g\ l^{-1}$ (Giroux, 2015), 0.12 $\mu g\ l^{-1}$ (Reupert and Ploeger, 1989) and 5.60 $\mu g\ l^{-1}$, (Quednow and Püttmann, 2007), respectively, being reported. By evaluating the effects of temperature (18, 21, 24 and 28 °C) and two concentrations of an equitoxic mixture of diuron (5.36 or 32.63 $\mu g\ l^{-1}$), isoproturon (4.74 or 28.87 $\mu g\ l^{-1}$), atrazine (4.96 or 30.19 $\mu g\ l^{-1}$), and terbutryn (5.55 or 33.78 $\mu g\ l^{-1}$) on freshwater periphytic algae, Larras et al. (2013) noted lower sensitivities of the diatoms when grown at higher temperatures. Similarly, Tasmin et al. (2014a,b) observed that rising water temperatures (from 10 to 30°C) reduced the toxicity of diuron (up to 32 $\mu g\ l^{-1}$) on the green algae *Pseudokirchneriella subcapitata*. This could be due to the smaller biovolumes of these organisms compared to the other species and to their decreased herbicide uptake rates (Tang et al., 1998; Larras et al., 2013). As seen with herbicides, decreased biovolumes will also limit nutrient uptake in diatoms—so that additional tolerance mechanisms involving better nutrient recycling, or specialized mechanisms of nutrient uptake with herbicide avoidance, may also be involved. In this context, it is important to note that temperature modulation of lipid composition is necessary to maintain membrane fluidity. This acclimation is important to assure efficient photosynthetic electron transport (which is dependent upon membrane-protein interactions) (Morgan-Kiss and Dolhi, 2012). Changes in membrane composition, however, can also drive changes in membrane permeability, reducing or increasing the penetration of pollutants into the cells (Tuckey et al., 2002), thus modulating their inhibitory effects on photosynthetic aquatic organisms.

Case of Glyphosate and Temperature

The introduction of glyphosate-resistant (GR) plants (Coupe et al., 2012) has resulted in glyphosate [*N*-(phosphonomethyl)glycine) becoming the most widely used herbicide worldwide. Due to its high water solubility and extensive use, glyphosate exposure of non-target aquatic organisms is a growing concern (Tsui and Chu, 2003). While glyphosate has been detected in many aquatic systems it rarely exceeds a few micrograms per liter (Pesce et al., 2008, 2009), which could be related to its rapid degradation to its major metabolite, aminomethylphosphonic acid (AMPA) (Kolpin et al., 2006; Borggaard and Gimsing, 2008). In surface waters, concentrations ranging from 1 to 50 μg glyphosate l^{-1} and from 4 to 48.9 μg AMPA l^{-1} were reported in Europe (Horth and Blackmore, 2009), while in Canada, concentrations observed were $\leq$ 40.8 and $\leq$ 1.1 $\mu g\ l^{-1}$ for glyphosate and AMPA, respectively (Giroux, 2015). AMPA has been identified as toxic to higher plants (Reddy et al., 2004), and was shown to disrupt chlorophyll biosynthesis (Gomes et al., 2016); its effects on algae and cyanobacteria, however, have not been thoroughly investigated.

Algal and cyanobacterial tolerance to glyphosate can vary among the species, but phytoplankton are generally considered tolerant to this herbicide (Wong, 2000; Tsui and Chu, 2003; Arunakumara et al., 2013; Wang et al., 2016). Glyphosate and AMPA have been shown to be degraded by cyanobacteria activity in aqueous environments (as reviewed by Arunakumara et al., 2013), and glyphosate mineralization could be related to the low glyphosate sensitivity of phytoplankton. Stachowski-Haberkorn et al. (2008) observed shifts in microbial diversity in *in situ* microcosms in a coastal ecosystem treated with low concentrations of Roundup ($<$10 $\mu g\ l^{-1}$). Glyphosate (e.g., at 150 $\mu g\ kg^{-1}$ of sediment dry weight) also affected freshwater sediment microbial communities (Widenfalk et al., 2008). Some toxic effects of a commercial formulation of glyphosate (Factor 540—at 50 $\mu g\ l^{-1}$ of glyphosate) on photosynthesis and growth were recently demonstrated for various phytoplankton species (Smedbol et al., under revision). These observations suggest that glyphosate exposure may affect photosynthetic aquatic organism communities, possibly by a glyphosate herbicidal mode of action or through glyphosate mineralization. Additionally, the growth of species able to degrade glyphosate and AMPA may be stimulated, as glyphosate mineralization represents a potential source of nutritional phosphorus and nitrogen (Widenfalk et al., 2008; Wang et al., 2016).

The detrimental effects of glyphosate are due to its inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (an enzyme of the shikimate pathway), which prevents the biosynthesis of aromatic amino acids (Siehl, 1997), leading to the reduction of protein synthesis. It has also been reported that glyphosate has detrimental effects on photosynthetic processes

that could be directly or indirectly linked to glyphosate-induced reductions of plastoquinone (PQ) biosynthesis (Cobb and Reade, 2010; Gomes et al., 2017b) and chlorophyll concentrations (Gomes et al., 2016). Reduction of PQ and chlorophyll contents will directly affect the chloroplast electron transport rate (ETR). Additionally, PQ is a co-factor of phytoene desaturase and ζ-carotene desaturase, during carotenoid synthesis (Sandmann et al., 2006), and decreased PQ content could therefore decrease cell carotenoid and xanthophyll contents [as β-carotene is known to be the precursor of the xanthophyll cycle (Bouvier et al., 1996)]. This would result in decreased thermal dissipation of excess energy, leading to ROS accumulation and oxidative bursts that would ultimately induce decreases in photosynthetic activity (**Figure 1**). Glyphosate was also proposed to affect photosynthesis by the overproduction of ROS in mitochondria due to its inhibitory effect on respiratory electron transport chain; once produced in mitochondria, ROS leave mitochondria, entering in chloroplast where they inducing oxidative damages to photosynthetic apparatus, decreasing photosynthesis (Gomes and Juneau, 2016).

As with other herbicides, glyphosate toxicity in the environment can be modulated by climatic conditions, especially by temperature (Pesce et al., 2009). However, studies on algae and cyanobacteria involving combined glyphosate and temperature effects are quite scarce. Pesce et al. (2009) demonstrated that glyphosate (10 μg l^{-1}) effects on riverine microorganisms are seasonally dependent, with no difference in algal community genera distributions being observed between control and treated microcosms in the spring, although there were marked differences between the algal communities in the summer, with the disappearance of three algal genera (*Asterionella*, *Cyclotella,* and *Oocystis*) in glyphosate-treated microcosms. Although differences occurred in the species found between the two seasons, they noticed that glyphosate had no effect on chlorophyll *a* concentrations. While glyphosate did not affect total community biomass, it had inhibitory effects on the reproduction of *Asterionella*, *Cyclotella,* and *Oocystis* during the summer (Pesce et al., 2009), indicating that glyphosate-tolerant species became more representative in the community biomass. More recently, Baier et al. (2016) observed reduced algal diversity (Shannon- and Evenness-index) by glyphosate (1.5, 3, and 4 mg l^{-1}) under increased temperatures. This suggests that responses of natural communities to glyphosate can vary with changing environmental conditions, and that temperature can modulate glyphosate toxicity in algae (Pesce et al., 2009).

As mentioned previously, glyphosate was proposed to impair respiration by inhibiting the mitochondrial electron transport chain, leading to direct ROS formation (Gomes and Juneau, 2016; Gomes et al., 2017a). As higher temperature stimulates respiration (Falkowski and Raven, 2013), decreasing metabolic rates under lower temperatures can lead to decreased ROS production, mainly through the slowing of respiratory

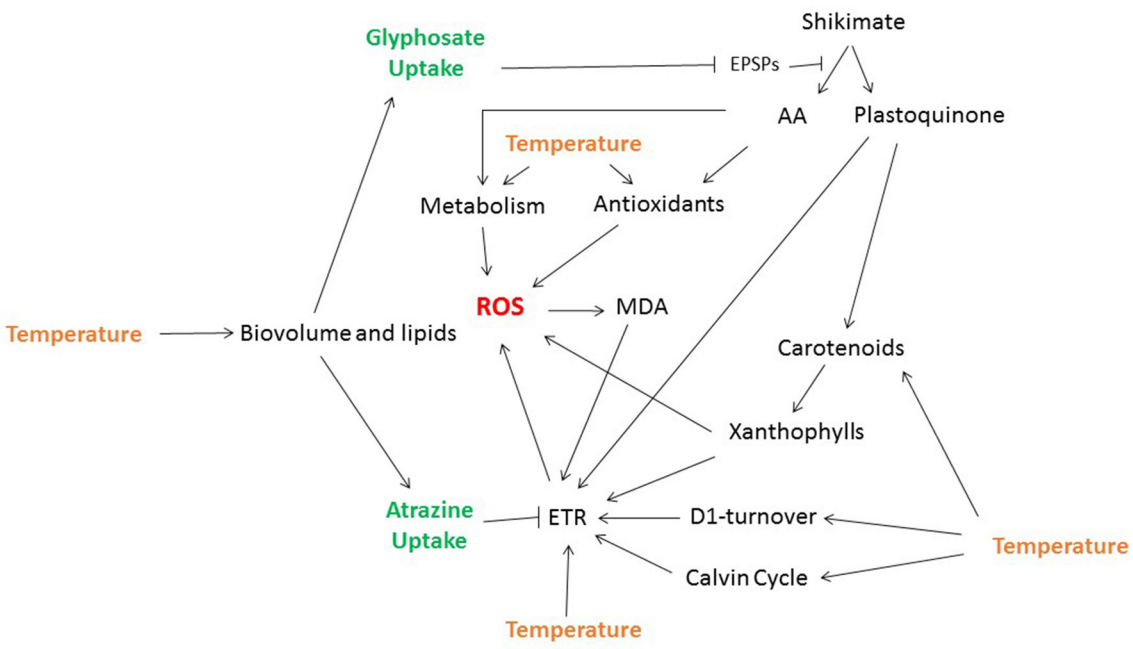


FIGURE 1 | Effects of temperature on the toxic effects of glyphosate and atrazine on photosynthetic aquatic organisms. Both glyphosate and atrazine can induce reactive oxygen species (ROS) accumulation, which will increase lipid peroxidation (MDA) and lead to oxidative bursts and membrane destruction (thylakoids), thus contributing to decreases in photosynthetic rates (ETR). Temperature can modulate the deleterious effects of atrazine and glyphosate exposure by: (1) inducing changes in cell biovolumes and lipid composition, and therefore herbicide uptake; (2) modulating enzymatic activity, including antioxidant systems and cell respiratory metabolism (which are both related to ROS content), as well as D1-protein recovery and the Calvin Cycle (which are involved in ETR and photosynthetic rates); (3) modulating pigment contents, especially of carotenoids, which are linked to thermal dissipation capacity, ROS, and the synthesis of xanthophylls; and (4) modulating membrane fluidity and the diffusion of electron carriers (which are related to ETR).

pathways. It is important to note, however, that temperature might modulate the activity of antioxidant systems, which are directly related to the control of ROS amounts in cells. The decreased activity of enzymatic antioxidant systems under low temperatures can lead to a less efficient ROS scavenging system, leading to their accumulation and deleterious effects (**Figure 1**). In contrast, although the increase in respiration could lead to increased ROS formation in glyphosate-exposed cells (due to increased glyphosate uptake and respiration activity), the increased activity of antioxidant system under physiological higher temperatures could prevent increases in the oxidative status (**Figures 1**, **2**).

Herbicides such as atrazine (as well other photosynthesis-targeting herbicides) and glyphosate are known to induce oxidative stress in photosynthetic aquatic organisms (DeLorenzo et al., 2001; Romero et al., 2011). It was shown that different species have various susceptibility to oxidative stress and thermal acclimation ability of the antioxidant defenses (Choo et al., 2004; Lohrmann et al., 2004; Bouchard and Purdie, 2011). It is known that the cyanobacteria *M. aeruginosa* decreases its metabolic activity when temperature is increased to a level that is considered stressful (Jochem, 1999). This may explain the decreased oxidative stress (ROS $cell^{-1}$) when *M. aeruginosa* cultures were transferred from 25 to 35°C (Bouchard and Purdie, 2011). In contrast, higher lipid peroxidation was observed at 15°C when compared to 26°C in the green algae *Cladophora glomerata* and *Enteromorpha ahlneriana*, which could be related to temperature modulation of their antioxidant enzymes since decreased ascorbate peroxidase (APX) activity was, in fact, observed when these algae were grown at 15°C (Choo et al., 2004). The modulation of antioxidant systems may therefore also be related to algal and cyanobacterial tolerance to herbicides under changing temperature regimes, although this important physiological process has not yet gained much attention, and inter-connected physiological evaluations of experimental results are missing. Xanthophylls, for example, is involved in the thermal dissipation of excess energy—while also contributing to ROS scavenging and increasing membrane stability by decreasing membrane susceptibility to lipid peroxidation (Havaux, 1998). In this context, pigment (carotenoid) content (as proposed by Millie et al., 1992), thermal dissipation (as proposed by Chalifour and Juneau, 2011), and oxidative stress could all be linked to the temperature modulation of herbicide toxicity in algae and cyanobacteria (**Figure 2**).

Temperature and herbicides could have both antagonistic and synergistic effects on photosynthetic aquatic organisms. Furthermore, temperature modulates uptake and metabolism of pollutants, which have important applied implications in respect to removal and fate of these pollutants in aquatic systems. Raising temperatures can significantly favor their removal by more tolerant algal and cyanobacterial species (which is of interest for reclaiming projects) but will drastically increase herbicide

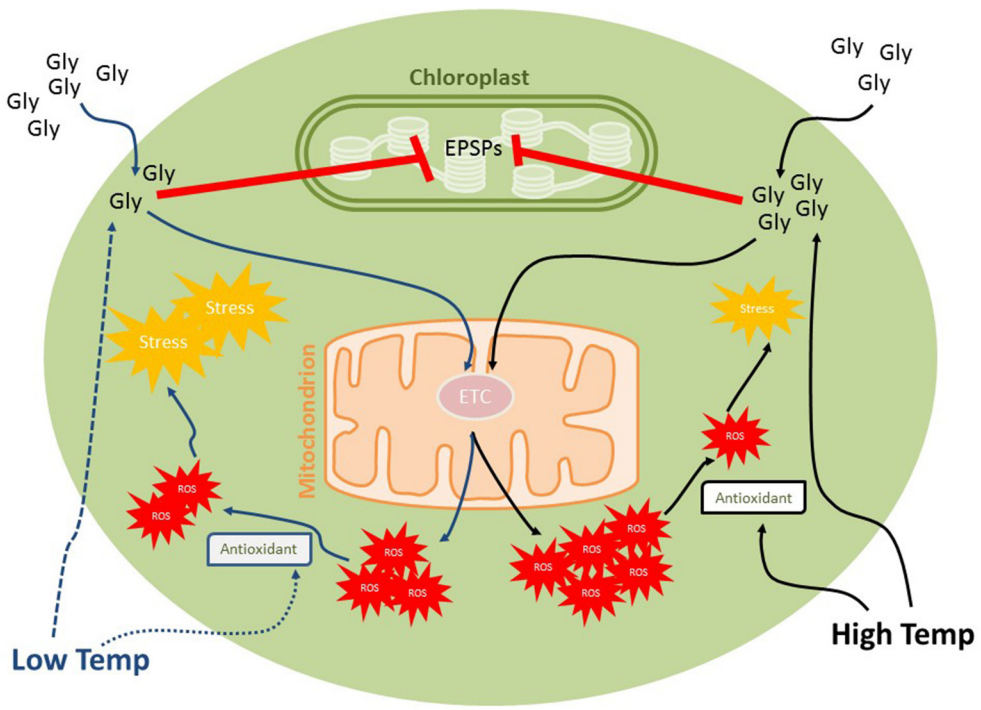


FIGURE 2 | Effects of low temperature and high physiological temperatures on the toxic effects of glyphosate on phytoplankton. Glyphosate herbicidal effects are due the inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase activity in chloroplast but it is also known to block mitochondrial electron transport chain, inducing reactive oxygen species (ROS) formation. Low temperature decrease both glyphosate uptake (by modulating membrane permeability) and antioxidant activities, the contrast being expected under increased temperatures. Although increasing glyphosate uptake, respiration activity and ROS formation, higher temperatures can induce antioxidant activities, decreasing potential oxidative damages from ROS accumulation.

toxicity to sensitive species, leading to possible irreversible changes in photosynthetic aquatic community.

LIGHT AND HERBICIDES IN PHYTOPLANKTON AND PERIPHYTON

General Concern

Phytoplankton success in aquatic environments is linked to their ability to convert light into biochemical energy that can be used in biomass production (Deblois et al., 2013). Photosynthesis is directly related to photon flux: at the light compensation point, CO_2 input (carboxylation) is equal to CO_2 release (respiration); above the light compensation point, increasing photon fluxes will increase photosynthetic rates in a linear relationship until reaching a saturating point at which factors other than incident light (i.e., the electron transport rate, Rubisco activity, or carbon metabolism) become limiting to photosynthesis. Therefore, photosynthetically active light can be a critical limiting resource for algal and cyanobacterial growth and reproduction.

Predictions of regional or global changes in light availabilities are currently difficult as (in addition to physical characteristics of water) light regimes will vary as a function of photosynthetic aquatic organism growth (Finkel et al., 2010). The new climatic scenario, however, is set to impose increasing light intensity and solar ultraviolet (UV) radiation on ecosystems due to ozone depletion (Caldwell et al., 2003) that can result in cellular damage and inhibitory effects on photosynthesis, at least in the upper water column (Finkel et al., 2010). Additionally, global warming will alter circulation patterns, water column stratification, and water layer mixing, resulting in algal and cyanobacterial exposure to high light and UV intensities (Finkel et al., 2010). In this context, predicted changes in light conditions will probably have an impact on the selection of species possessing strategies to cope with or avoid photoinhibition (Sunda et al., 2002).

Agricultural wastes increase water turbidity and decrease light availability for photosynthetic aquatic community (Tilman et al., 2001). Additionally, wind episodes may cause photosynthetic organisms to be exposed to strong light intensity variations during the day (Dubinsky and Stambler, 2009). In these conditions, algae and cyanobacteria may alter their cellular metabolism and invest more resources to produce UV absorbing compounds (to shield UV-sensitive cellular components) and to increase the metabolic repair processes of PSII after photoinactivation (Finkel et al., 2010; Falkowski and Raven, 2013). Photoacclimation processes can also alter algal and cyanobacterial cell sizes, as light-limiting environments often result in decreased cell sizes (which help to assure less internal light shading and thus higher light absorption efficiency), where under higher light conditions larger cells are observed (Finkel et al., 2010). Changes in cellular sizes can also be related to the uptake of pollutants by aquatic organisms, with decreased cell sizes being directly related to decreased contaminant uptake. Additionally, and at same time, once exposed to higher light conditions, small cells are more prone to photoinhibition (Falkowski and Raven, 2013)—making studies of the effects of water contaminants on algae and cyanobacteria under changing light environments essential.

Case of PSII Inhibitors and Light

Light stress targets the photosynthetic apparatus, as do some herbicides, and photoacclimation processes may therefore directly influence herbicide toxicity in aquatic photosynthetic organisms. The first studies examining the relationships between light and herbicides date from the 1980s (O'Neal and Lembi, 1983; Mayasich et al., 1986). O'Neal and Lembi (1983) observed that the effectiveness of the herbicide simazine (a triazine herbicide often used to control algal growth in USA and Europe) was influenced by light intensity. These authors found that the inhibitory effects of simazine (0.76 $\mu g\ l^{-1}$) on *C. glomerata* growth (a filamentous algae) increased with increasing light intensity. Similar results were also reported by Millie et al. (1992), who observed that the deleterious effects of simazine (20, 40, 80, 160, and 320 $\mu g\ l^{-1}$) on photosynthesis in *A. circinalis* became even greater in photoacclimated populations as photon flux density increased (50, 130, 230 $\mu mol\ m^{-2}\ s^{-1}$). In this context, the algaecide efficiency of simazine should increase under higher light conditions but be more limited in shaded environments. Millie et al. (1992) demonstrated that increases in simazine-toxicity for high light photoacclimated *A. circinalis* were correlated with reductions in pigment contents (chlorophyll *a*, *c*-phycocianin, and carotenoids), leading the authors to suggest that algal sensitivity to PSII inhibitors is affected by alterations in their pigment contents. In their study of the interactive effects of light (0.208, 0.780, and 1.352 mW/cm^2) and atrazine (also a triazine herbicide—0.05 and 0.10 $\mu g\ l^{-1}$) on the marine algae *Phaeodactylum tricornutum*, Mayasich et al. (1986) observed that the inhibitory effects of this herbicide were greater at low light intensities. These authors hypothesized that cells pre-acclimated to higher light acquired protective mechanisms of sufficient capacity to dissipate atrazine-induced photo-oxidative stress by diverting the excitation energy from PSII to PSI and/or through quenching by carotenoid pigments. Although increases in accessory pigments have been observed in low-light photoacclimated cells (Guasch and Sabater, 1998), these authors did not quantify their pigment contents to confirm that hypothesis. It is important to note that other mechanisms besides pigment modulation (i.e., increased antioxidant system efficiency) could be involved in the influence of light on photosynthesis-inhibiting herbicides.

In their studies (using series of eight concentrations from 0 to 4.31 $mg\ l^{-1}$) of changes in atrazine toxicity during succession in stream periphyton communities, Guasch et al. (1997) observed that communities photoacclimated to higher irradiance levels (150 as opposed to 50 $\mu mol\ m^{-2}\ s^{-1}$) showed a lower photosynthetic EC_{50} (atrazine concentrations inhibiting 50% of the photosynthetic rate)—suggesting a relationship between light conditions during growth and atrazine toxicity in which aquatic photosynthetic organism life histories have a central role in their responses to atrazine. Guasch and Sabater (1998) confirmed this hypothesis in subsequent short-term concentration-response test studies, reporting that periphyton communities that colonized shaded sites showed higher percentages of accessory pigments and were less sensitive (higher photosynthetic EC_{50}) to atrazine than communities from open sites (photoacclimated to higher

light intensities), indicating that this herbicide was more toxic to high light-photoacclimated algae.

The importance of the light histories of phytoplankton to their responses to atrazine (from 0.005 to 2.156 mg l^{-1}) was also tested by Deblois et al. (2013) who examined the combined effects of different light intensities and atrazine concentrations on 10 phytoplankton species (chlorophytes, baccilariophytes, and cyanophytes) adapted or not to low [LL – 76 µmol photons (PAR) m^{-2} s^{-1}] or high [HL – 583 µmol photons (PAR) m^{-2} s^{-1}] light intensities. Firstly, these authors observed that cyanophytes were more sensitive to atrazine, indicating a species-dependent response to the herbicide. Additionally, evaluations of photosynthetic parameters indicated a positive relationship between atrazine toxicity and increased irradiance for non-adapted phytoplankton and these authors argued that this relationship could be related to changes affecting electron transport rates due to rapid photoregulatory processes. The availability of light and electron transporters (such as quinones) were advance to be the limiting factors under LL and HL situations, respectively. Indeed, under LL condition and fixed atrazine concentration only a small fraction of the quinone pool needed for photosynthesis would therefore be blocked, attenuating the deleterious effects of the herbicide. In contrast, under HL conditions the entire quinone pool is needed for effective electron transport to avoid photoinhibition, and atrazine would therefore have a more deleterious effect by further limiting the electron transport (and increasing photoinhibitory effect). These authors also observed that for HL-adapted organisms atrazine has less effect than for the LL-adapted phytoplankton. Deblois et al. (2013) noted that HL-adaptation often increases the size of the electron transport pool (including quinones), increasing the quantity of target sites of atrazine and thus diluting its hazardous effects (**Figure 3**). The results of Deblois et al. (2013) on adapted algae and cyanobacteria, therefore, seem to contrast with those of Guasch and Sabater (1998). However, it is important to note that these authors were working with different levels of biological complexity: while Guasch and Sabater (1998) evaluated the atrazine responses of a periphyton community, Deblois et al. (2013) investigated herbicide effects at the level of individual phytoplankton species. Furthermore, when we take into account that the HL-acclimated (and high atrazine sensitive) community studied by Guasch and Sabater (1998) was predominantly composed of chlorophytes and cyanophytes, the results of Deblois et al. (2013) showing that cyanophytes were more sensitive to atrazine than other phytoplankton groups, are in accordance with the community level study.

Case of Lipid Synthesis Inhibitor and Light

Molinate (a thiocarbamate herbicide) is widely used in China in rice plantations in both pre- and post-emergence applications (Yan et al., 1997). Yan et al. (1997) evaluated the effects of molinate (5, 25, and 50 µg l^{-1}) on *Anabaena sphaerica*, a filamentous nitrogen-fixing cyanobacterium, and reported that its toxicity was higher under high light conditions (3000 lux

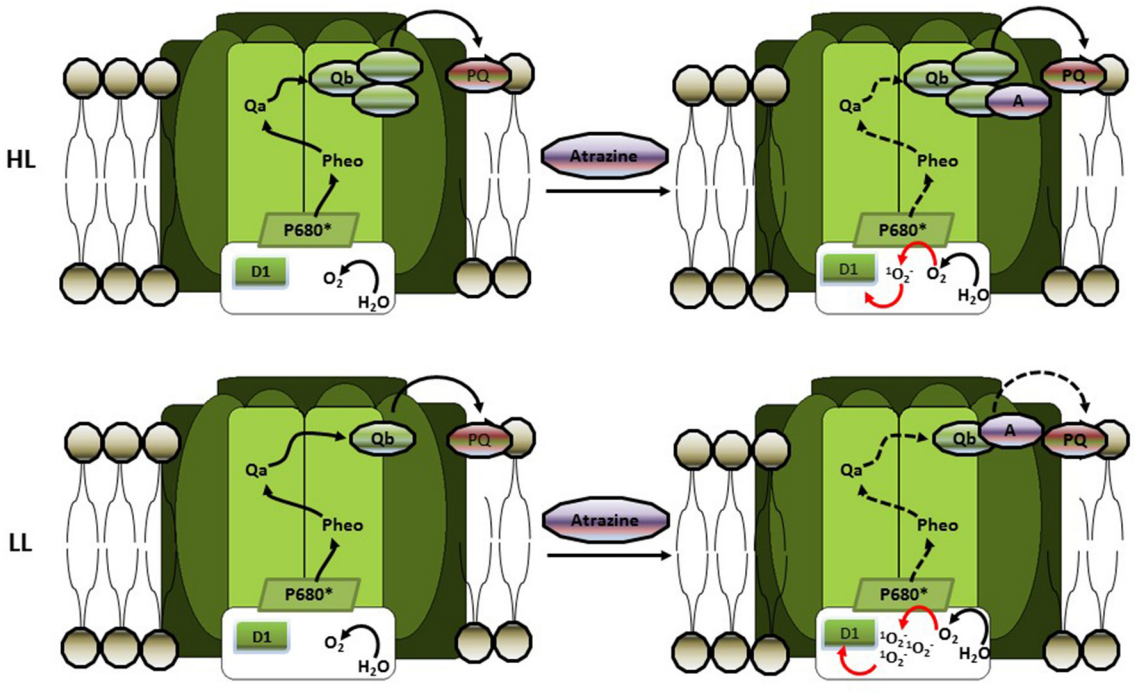


FIGURE 3 | The influence of high light (HL) and low light (LL) acclimation on atrazine toxicity in phytoplankton (according to Deblois et al., 2013). HL-adaptation increases the quinone pool, thus increasing the number of atrazine targets. This dilution effect decreases reactive oxygen production (ROS) due to atrazine-induced photoinhibition, thus decreasing the hazardous effects of that herbicide. In LL adapted cells, on the other hand, the lack of sufficient quinone to maintain electron transport will drastically increase (ROS) production and oxidative damage.

compared to 300 lux). Interestingly, molinate was observed to stimulate chlorophyll *a* biosynthesis in *A. sphaerica,* although the stimulatory effect was lower under lower light conditions (300 lux) than at 3000 lux. On the other hand, molinate interfered with *A. sphaerica* protein metabolism, inhibiting the biosynthesis of biliproteins that are important light-harvesting components in cyanobacteria, and molinate toxicity was greater under high light intensity conditions due to decreased organic carbon assimilation (which is known to chelate the herbicide to form an inactive complex, thus diminishing its toxicity).

Case of Glyphosate and Light

In spite of the environmental importance of glyphosate, to our knowledge, only one study examining light effects on its toxicity in photosynthetic aquatic organisms was published. In their study, Wood et al. (2016) examined the effects of changing light intensities (20 and 100 μmol m^{-2} s^{-1}) on glyphosate (50, 200, and 500 $\mu g\,l^{-1}$) toxicity to natural benthic communities collected from 4 different rivers in the Great Barrier Reef World Heritage Area (Australia). For the majority of the evaluated taxa (22 of the 26) and for the entire community, no significant interaction between light and herbicide effects at the community levels was found. It is important to note, however, that the response of diatoms to herbicide exposure at the community levels was dependent on prior exposure histories of the sites and can be influenced by intra-specific interactions between taxa (Wood et al., 2016).

Under a physiological point of view, interaction effects between glyphosate and light conditions can be expected since light modulates the quantities of electron transport components such as plastoquinones that are produced in the shikimate pathway—the target of the herbicide glyphosate. Shick et al. (1999) demonstrated that both UV-A and B radiation stimulate the shikimate pathway, and they evaluated the mycosporine-like amino acid (MAA) contents of the algal partners of colonies of *Stylophorapistillata* coral that are produced via the shikimate pathway (Bandaranayake, 1998). Exposure to UV was found to induce MAA accumulation, indicating that the shikimate pathway was stimulated by UV irradiation (Bandaranayake, 1998). In this context, increased environmental UV could result in either increased or decreased glyphosate toxicity: (1) by inhibiting EPSPs, as glyphosate induces unregulated carbon flux through shikimate pathway, thus disturbing carbon metabolism and several cellular metabolic pathways (Gomes et al., 2014). Stimulation of the shikimate pathway by UV exposure could therefore increase the negative impact of glyphosate on cell metabolism; (2) by stimulating the shikimate pathway, as previous exposure to UV can increase aromatic amino acid production (the first physiological glyphosate target) and plastoquinone (the secondary physiological glyphosate target) contents of the cells, decreasing the detrimental effects of glyphosate on protein biosynthesis and photosynthesis respectively (for more details see **Figure 1**). Since studies on the interactions between light and glyphosate are at their infancy, additional studies are definitively needed.

Changing light environments can act antagonistically or synergistically with herbicides effects on phytoplankton physiology. Although light-limiting conditions could decreased chemical uptake by reducing cell sizes, it may favor negative effect of photosynthetic-target herbicides in aquatic organisms (when compared to cells photoacclimated to higher light intensities) due to less effective protective mechanisms associated to pigment contents. In this context, we can expect that the increase in light intensities could favor the presence of algal and cyanobacterial species less sensitive to PSII inhibitors (such as atrazine) due to the presence of species having more efficient photoacclimation mechanisms.

FUTURE PERSPECTIVES AND CONCLUSIONS

Changes in temperature and light regimes exert profound effects on phytoplankton metabolic processes as well as on the environment. Therefore, the physiological responses of phytoplankton to aquatic pollutants would be expected to be modulated by environmental changes. Guidelines for the protection of aquatic life have largely been restricted until now to studies carried out at temperatures between 20 and 30°C (USEPA, 2004; MDDEP, 2008). New environmental pressures (mainly anthropological) are currently driving changes in environmental conditions, however, especially in terms of temperature and light conditions, studies directed toward the influence of environmental factors on aquatic pollutant toxicity are urgently needed. The use of herbicides has greatly increased over the last few decades, and herbicide contamination, especially of aquatic systems, is a growing concern. Most herbicides (such as atrazine) accumulate in aquatic ecosystems; others (such as glyphosate) are quickly degraded, although the toxicities of their by-products have not been extensively studied. The glyphosate by-product AMPA, for example, has been considered toxic to plants (Gomes et al., 2016), but no studies of its effects on phytoplankton have been undertaken yet. This presents a double blind spot in the literature: (1) our lack of knowledge concerning the toxicities of pollutants (including some herbicides and large numbers of herbicide by-products) and, (2) our lack of knowledge about how environmental changes can affect herbicide toxicities in aquatic ecosystems.

In attempting to address these questions, a number of authors have conducted studies examining the influences of temperature and light on herbicide toxicity in phytoplankton, but their results have not been consistent or conclusive. Firstly, the duration of phytoplankton acclimation processes have varied among the studies (from a few hours to several days) and no study has compared temperature or light effects on herbicide toxicity over short- vs. long-term timescales. Acclimation processes are of great importance to phytoplankton metabolism, and acclimation histories are central to understand phytoplanktonic responses to herbicides (Guasch and Sabater, 1998). The duration of phytoplankton acclimation can therefore influence their responses to waterborne contaminants, and this point must be considered when comparing new studies to established models. Secondly, studies have often only used the metrics of growth and photosynthesis to evaluate phytoplankton responses to herbicide exposure under changing environmental conditions. Despite their obvious importance, growth and photosynthesis are not

the only physiological aspects that can or should be considered. Many herbicides can induce oxidative stress, although oxidative mechanisms (such as the activities of antioxidant systems, ROS accumulation, and lipid peroxidation) have not been widely used as toxicity markers. Oxidative events are central features in metabolism, as ROS can induce cell damage but also act as cellular signaling molecules (Gomes and Garcia, 2013). Investigations of these processes can help us to elucidate herbicide toxicity and identify the effects of herbicides on phytoplankton metabolism in an interconnected manner.

It is difficult to predict the effects of environmental changes on herbicide toxicity to phytoplankton as temperature and light can modulate phytoplankton responses to herbicides through acclimation processes. The changes induced at the metabolic machinery level by these climatic factors can either increase or decrease phytoplankton tolerance (even in a species-specific manner). These responses are highly specific and can vary between strains of the same species (Chalifour and Juneau, 2011). Temperature could possibly modulate the deleterious effects of herbicides such as atrazine and glyphosate by: (1) inducing changes in cell biovolumes and their lipid compositions, and therefore herbicide uptake; (2) modulating enzymatic activities, including those of antioxidant systems and of cell respiratory metabolism (which are both related to ROS content), as well as D1-protein recovery and the Calvin Cycle (which are involved in ETR and photosynthetic rates); (3) modulating the pigment contents of the cells, especially carotenoids, which are linked to thermal energy dissipation capacity, ROS scavenging, and synthesis of xanthophylls; and (4) modulating membrane fluidity and the diffusion of electron carriers (which are related to ETR). On the other hand, light appears to influence algal and cyanobacterial responses to herbicides (at least those that target photosynthesis) by modulating the numbers of electron transporters (such as quinones) and by increasing or decreasing the concentrations of herbicide binding sites (Deblois et al., 2013). By modulating metabolic changes (acclimation), both temperature and light can drive herbicide tolerance and will be important factors in selecting species able to survive to herbicide contamination. In this context, total primary production may not be affected by the presence of herbicides in aquatic environments although phytoplankton biodiversity will certainly be altered (as observed by Pesce et al., 2009). In addition to reducing biodiversity, these interactions can induce eutrophication processes by stimulating herbicide mineralization, for example, or by increasing the nutrient contents of waterways (Stachowski-Haberkorn et al., 2008).

Among the studied environmental factors, temperature seems to be the most important in driving phytoplankton responses to herbicide contamination. Considering the present literature, we can advise that the season of herbicide application might influence the phytoplankton community in the following sense: at low temperatures, herbicide application may reduce cyanobacterial community, since these species are quite sensitive to low temperature (Butterwick et al., 2004). On the other hand, at warmer temperatures, cyanobacteria may have higher tolerance to the hazardous effects of herbicides, and their growth may be favored compared to other groups of algae and cyanobacteria. Since many cyanobacteria can produce harmful toxins, their dominance induce, on top of herbicide presence, another environmental problem. In this context, the use of herbicide during the summer (or in equatorial and tropical regions) might represent an additional ecological constrain, as it can induce hazardous cyanobacterial blooms. In turns, light conditions appear to be more linked to herbicide efficiency, as found for the algaecide efficiency of simazine (Millie et al., 1992). However, it is important to consider that ozone depletion will induce increased UV radiation, which influences phytoplankton performance. However, few studies have been conducted regarding the interaction between UV and herbicides.

As an overview, the published literature emphasizes the importance of light regimes and temperatures on herbicide toxicity to algae and cyanobacteria—although it is difficult today to establish species-specific models of how climate change will affect phytoplanktonic and periphytonic responses to herbicides. Acclimation processes will affect the selection of algal and cyanobacterial species able to survive under conditions of herbicide contamination, assuring sustained primary production, although this process will necessarily decrease biodiversity. More investigations are needed to determine how these interactions will influence the success of organisms of different taxa, and it will be important to include environmental changes as variables in any new guidelines proposed for the protection of aquatic life forms. Finally, guidelines should be re-evaluated based on field studies, instead of being developed based on only *in situ* evaluations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This research was supported by the Natural Science and Engineering Research Council of Canada (NSERC). MPG received a Ph.D. scholarship from Fond de Recherche du Quebec–Nature et Technologies (FRQNT).

REFERENCES

Arunakumara, K. K. I. U., Walpola, B. C., and Yoon, M. (2013). Metabolism and degradation of glyphosate in aquatic cyanobacteria : a review. *Afr. J. Microbiol. Res.* 7, 4084–4090. doi: 10.5897/AJMR12.2302

Atkin, O. K., and Tjoelker, M. G. (2003). Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends Plant Sci.* 8, 343–351. doi: 10.1016/S1360-1385(03)00136-5

Baier, F., Gruber, E., Hein, T., Bondar-kunze, E., Ivankovic, M., and Mentler, A. (2016). Non-target effects of a glyphosate-based herbicide on Common Toad larvae (Bufo bufo, Amphibia) and associated algae are altered by temperature. *PeerJ* 4:e2641. doi: 10.7717/peerj.2641

Bandaranayake, W. M. (1998). Mycosporines: Are they nature's sunscreens? *Nat. Prod. Rep.* 15, 159–172. doi: 10.1039/a815159y

Barber, J., Ford, R. C., Mitchell, R. A. C., and Millner, P. A. (1984). Chloroplast thylakoid membrane fluidity and its sensitivity to temperature. *Planta* 161, 375–380. doi: 10.1007/BF00398729

Baxter, L., Brain, R. A., Lissemore, L., Solomon, K. R., Hanson, M. L., and Prosser, R. S. (2016). Influence of light, nutrients, and temperature on the toxicity of atrazine to the algal species *Raphidocelis subcapitata*: implications for the risk assessment of herbicides. *Ecotoxicol. Environ. Saf.* 132, 250–259. doi: 10.1016/j.ecoenv.2016.06.022

Behrenfeld, M. J., Prasil, O., Babin, M., and Bruyant, F. (2004). In search of a physiological basis for covariations in light-limited and light saturated photosynthesis. *J. Phycol.* 40, 4–25. doi: 10.1046/j.1529-8817.2004.03083.x

Bérard, A., and Benninghoff, C. (2001). Pollution-induced community tolerance (PICT) and seasonal variations in the sensitivity of phytoplankton to atrazine in nanocosms. *Chemosphere* 45, 427–437. doi: 10.1016/S0045-6535(01)00063-7

Bérard, A., Leboulanger, C., and Pelte, T. (1999). Tolerance of oscillatoria limnetica lemmermann to atrazine in natural phytoplankton populations and in pure culture : influence of season and temperature. *Arch. Environ. Contam. Toxicol.* 479, 472–479. doi: 10.1007/s002449900541

Bicalho, E. M., Gomes, M. P., Rodrigues, A. G. Jr., Oliveira, T. G. S., de Almeida Gonçalves, C., Fonseca, M. B., et al. (2017). Integrative effects of zinc and temperature on germination in *Dimorphandra wilsonii* rizz.: implications of climate changes. *Environ. Toxicol. Chem.* 9999, 1–7. doi: 10.1002/etc.3729

Borggaard, O. K., and Gimsing, A. L. (2008). Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. *Pest Manag. Sci.* 456, 441–456. doi: 10.1002/ps.1512

Bouchard, J. N., and Purdie, D. A. (2011). Effect of elevated temperature, darkness, and hydrogen peroxide treatment on oxidative stress and cell death in the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* 1. *J. Phycol.* 47, 1316–1325. doi: 10.1111/j.1529-8817.2011.01074.x

Boussiba, S. (2000). Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. *Physiol. Plant.* 108, 111–117. doi: 10.1034/j.1399-3054.2000.108002111.x

Bouvier, F., D'Harlingues, A., Hugueney, P., Marin, E., Marion-Poll, A., and Camara, B. (1996). Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta -cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *J. Biol. Chem.* 271, 28861–28867.

Butterwick, C., Heaney, S. I., and Talling, J. F. (2004). Diversity in the influence of temperature on the growth rates of freshwater algae, and its ecological relevance. *Freshw. Biol.* 50, 291–300. doi: 10.1111/j.1365-2427.2004.01317.x

Caldwell, M. M., Ballaré, C. L., Bornman, J. F., Flint, S. D., Bjorn, L. O., Teramura, A. H., et al. (2003). Terrestrial ecosystems, increased solar ultraviolet radiation and interactions with other climatic change factors. *Photochem. Photobiol. Sci.* 2, 252–266. doi: 10.1039/B211159B

Carafa, R., Wollgast, J., Canuti, E., Ligthart, J., Dueri, S., Hanke, G., et al. (2007). Seasonal variations of selected herbicides and related metabolites in water, sediment, seaweed, and clams in the Sacca di Gorocoastal lagoon (Northern Adriatic). *Chemosphere* 69, 1625–1637. doi: 10.1016/j.chemosphere.2007.05.060

Chalifour, A., Arts, M. T., Kainz, M. J., and Juneau, P. (2014). Combined effect of temperature and bleaching herbicides on photosynthesis, pigment and fatty acid composition of *Chlamydomonas reinhardtii*. *Eur. J. Phycol.* 49, 508–515. doi: 10.1080/09670262.2014.977962

Chalifour, A., and Juneau, P. (2011). Temperature-dependent sensitivity of growth and photosynthesis of *Scenedesmus obliquus*, *Navicula pelliculosa* and two strains of *Microcystis aeruginosa* to the herbicide atrazine. *Aquat. Toxicol.* 103, 9–17. doi: 10.1016/j.aquatox.2011.01.016

Chen, C. Y., Hathaway, K. M., Thompson, D. G., and Folt, C. L. (2008). Multiple stressor effects of herbicide, pH, and food on wetland zooplankton and a larval amphibian. *Ecotoxicol. Environ. Saf.* 71, 209–218. doi: 10.1016/j.ecoenv.2007.08.007

Chen, M., Li, J., Dai, X., Sun, Y., and Chen, F. (2010). Effect of phosphorus and temperature on chlorophyll a contents and cell sizes of *Scenedesmus obliquus* and *Microcystis aeruginosa*. *Limnology* 12, 187–192. doi: 10.1007/s10201-010-0336-y

Choo, K., Snoeijs, P., and Pedersén, M. (2004). Oxidative stress tolerance in the filamentous green algae *Cladophora glomerata* and *Enteromorpha ahlneriana*. *J. Exp. Mar. Bio. Ecol.* 298, 111–123. doi: 10.1016/j.jembe.2003.08.007

Cobb, A. H., and Reade, J. P. (2010). "The inhibition of amino acid byosynthesis," in Herbicides and *Plant Physiology*, eds A. H. Cobb and J. P. Reade (Wiley-Blackwell), 176–197.

Coles, J. F., and Jones, R. C. (2000). Effect of temperature on photosynthesis-light response and growth of four phytoplankton species isolated from a tidal freshwater river. *J. Phycol.* 16, 7–16. doi: 10.1046/j.1529-8817.2000.98219.x

Comber, S. D. W. (1999). Abiotic persistence of atrazine and simazine in water. *Pestic. Sci.* 55, 696–702.

Corbett, J. R., Wright, K., and Baillie, A. C. (1984). *The Biochemical Mode of Action of Pesticides*. London: Academic.

Costanzoi, S. D., and Dennison, W. C. (2003). Assessing the seasonal influence of sewage and agricultural nutrient inputs in a subtropical river estuary. *Estuaries* 26, 857–865. doi: 10.1007/BF02803344

Coupe, R., Kalkhoff, S., Capel, P., and Gregoire, C. (2012). Factors affecting the fate and transport of glyphosate and AMPA into surface waters of agricultural watersheds in the United States and Europe. *Geophys. Res. Abstr.* 14:5877.

Crain, C. M., Kroeker, K., and Halpern, B. S. (2008). Interactive and cumulative effects of multiple human stressors in marine systems. *Ecol. Lett.* 11, 1304–1315. doi: 10.1111/j.1461-0248.2008.01253.x

Croll, B. (1991). Pesticides in surface and underground waters. *Water Environ. J.* 5, 389–395. doi: 10.1111/j.1747-6593.1991.tb00635.x

Cuypers, A., Vangronsveld, J., and Clijsters, H. (2001). The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of Phaseolus vulgaris. *Plant Physiol. Biochem.* 39, 657–664. doi: 10.1016/S0981-9428(01)01276-1

Daniel, R. M., Dines, M., and Peatach, H. H. (1996). The denaturation and degradation of stable enzymes at high temperatures. *Biochem. J.* 317, 1–11. doi: 10.1042/bj3170001

Davison, I. R. (1991). Environmental effects on algal photosynthesis: temperature. *J. Phycol.* 27, 2–8. doi: 10.1111/j.0022-3646.1991.00002.x

Debenest, T., Silvestre, J., Coste, M., and Pinelli, E. (2010). Effects of pesticides on freshwater diatoms. *Rev. Environ. Contam. Toxicol.* 203, 87–103. doi: 10.1007/978-1-4419-1352-4_2

Deblois, C. P., Dufresne, K., and Juneau, P. (2013). Response to variable light intensity in photoacclimated algae and cyanobacteria exposed to atrazine. *Aquat. Toxicol.* 126, 77–84. doi: 10.1016/j.aquatox.2012.09.005

DeLorenzo, M. E., Cott, G. I., and Ross, P. (2001). Toxicity of pesticides to aquatic microorganisms: a review. *Environ. Toxicol. Chem.* 20, 84–98. doi: 10.1002/etc.5620200108

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., et al. (2008). Impacts of climate warming on terrestrial ectotherms across latitude Thermal Safety margin. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6668–6672. doi: 10.1073/pnas.0709472105

Diana, S. G., Resetarits, W. J., Schaeffer, D. J., Beckmen, K. B., and Beasley, V. R. (2000). Effects of atrazine on amphibian growth and survival in artificial aquatic communities. *Environ. Toxicol. Chem.* 19, 2961–2967. doi: 10.1002/etc.5620191217

Dubinsky, Z., and Stambler, N. (2009). Photoacclimation processes in phytoplankton: mechanisms, consequences, and applications. *Aquat. Microb. Ecol.* 56, 163–176. doi: 10.3354/ame01345

Elliott, J. A. (2010). The seasonal sensitivity of Cyanobacteria and other phytoplankton to changes in flushing rate and water temperature. *Glob. Chang. Biol.* 16, 864–876. doi: 10.1111/j.1365-2486.2009.01998.x

Falkowski, P. G., and Raven, J. A. (2013). *Aquatic Photosynthesis*. 2nd Edn., Princeton, NJ: Princeton University Press.

Finkel, Z. V., Beardall, J., Flynn, K. J., Quigg, A., Rees, T. A. V., and Raven, J. A. (2010). Phytoplankton in a changing world: cell size and elemental stoichiometry. *J. Plankton Res.* 32, 119–137. doi: 10.1093/plankt/fbp098

Fiori, E., and Pistocchi, R. (2014). *Skeletonema marinoi* (Bacillariophyceae) sensitivity to herbicides and effects of temperature increase on cellular responses to terbuthylazine exposure. *Aquat. Toxicol.* 147, 112–120. doi: 10.1016/j.aquatox.2013.12.014

Folt, C. L., Chen, C. Y., Moore, M., and Burnaford, J. (1999). Synergism and antagonism among multiple stressors. *Limnol. Oceanogr.* 44, 854–877. doi: 10.4319/lo.1999.44.3_part_2.0864

Gilmore, A. M. (1997). Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. *Physiol. Plant.* 99, 197–209. doi: 10.1111/j.1399-3054.1997.tb03449.x

Giroux, I. (2015). *Présence de Pesticides dans l'eau au Québec : Portrait et Tendances Dans les Zones de maïs et de soya – 2011 à 2014, Ministère du Développement Durable, de l'Environnement et de la Lutte contre les changements climatiques*.

Gomes, M. P., Cruz, F. V. S., Bicalho, E. M., Borges, F. V., Fonseca, M. B., Juneau, P., et al. (2017a). Effects of glyphosate acid and the glyphosate-commercial formulation (Roundup) on *Dimorphandra wilsonii* seed germination: interference of seed respiratory metabolism. *Environ. Pollut.* 220, 452–459. doi: 10.1016/j.crvi.2005.08.004

Gomes, M. P., and Garcia, Q. S. (2013). Reactive oxygen species and seed germination. *Biologia* 68, 351–357. doi: 10.2478/s11756-013-0161-y

Gomes, M. P., and Juneau, P. (2016). Oxidative stress in duckweed (*Lemna minor* L.) induced by glyphosate: is the mitochondrial electron transport chain a target of this herbicide? *Environ. Pollut.* 218, 402–409. doi: 10.1016/j.envpol.2016.07.019

Gomes, M. P., Le Manac'h, S. G., Hénault-Éthier, L., Labrecque, M., Lucotte, M., and Juneau, P. (2017b). Glyphosate-dependent inhibition of photosynthesis in willow. *Front. Plant Sci.* 8:207. doi: 10.3389/fpls.2017.00207

Gomes, M. P., Le Manac'h, S. G., Maccario, S., Labrecque, M., Lucotte, M., and Juneau, P. (2016). Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll metabolism in willow plants. *Pestic. Biochem. Physiol.* 130, 65–70. doi: 10.1016/j.pestbp.2015.11.010

Gomes, M. P., Smedbol, E., Chalifour, A., Hénault-Ethier, L., Labrecque, M., Lepage, L., et al. (2014). Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid (AMPA), an overview. *J. Exp. Bot.* 65, 4691–4703. doi: 10.1093/jxb/eru269

Gonzáles-Barreiro, Ó., Rioboo, C., Cid, A., and Herrero, C. (2004). Atrazine-induces chlorosis in *Syechococcus elongatus* cells. *Arch. Environ. Contam. Toxicol.* 46, 301–307. doi: 10.1007/s00244-003-2149-z

Graymore, M., Stagnitti, F., and Allinson, G. (2001). Impacts of atrazine in aquatic ecosystems. *Environ. Int.* 26, 483–495. doi: 10.1016/S0160-4120(01)00031-9

Guasch, H., Mu, I., and Sabater, S. (1997). Changes in atrazine toxicity throughout succession of stream periphyton communities. *J. Appl. Phycol.* 9, 137–146. doi: 10.1023/A:1007970211549

Guasch, H., and Sabater, S. (1998). Light history influences the sensitivity to atrazine in periphytic algae. *J. Phycol.* 241, 233–241. doi: 10.1046/j.1529-8817.1998.340233.x

Haidekker, A., and Hering, D. (2008). Relationship between benthic insects (Ephemeroptera, Plecoptera, Coleoptera, Trichoptera) and temperature in small and medium-sized streams in Germany: a multivariate study. *Aquat. Ecol.* 42, 463–481. doi: 10.1007/s10452-007-9097-z

Hao, Y. B., Liu, H. L., Ci, X. K., Dong, S. T., Zhang, J. W., and Liu, P. (2010). Effects of arsenic on maize growth, antioxidant system, and ion distribution. *J. Appl. Ecol.* 21, 3183–3190.

Havaux, M. (1998). Carotenoids as membrane stabilizers in chloroplasts. *Trends Plant Sci.* 3, 147–151. doi: 10.1016/S1360-1385(98)01200-X

Herzig, R., and Dubinsky, Z. (1992). Photoacclimation, photosynthesis, and growth in phytoplankton. *Isr. J. Bot.* 41, 199–212.

Hölzl, G., and Dörmann, P. (2007). Structure and function of glycoglycerolipids in plants and bacteria. *Prog. Lipid Res.* 46, 225–243. doi: 10.1016/j.plipres.2007.05.001

Horth, H., and Blackmore, K. (2009). *Survey of Glyphosate and AMPA in Groundwaters and Surface Waters in Europe*. Final Report. WRc Ref: UC8073.02. Monsanto, Wiltshire, UK.

Huner, N. A., Öquist, G., and Melis, A. (2003). "Photostasis in plants, green algae and cyanobacteria: The role of light harvesting antenna complexes," in *Light-Harvesting Antennas in Photosynthesis SE - 14 Advances in Photosynthesis and Respiration*., eds B. Green and W. Parson (Dordrecht: Springer), 401–421.

IFEN (2006). *Pesticides in Water, 2003 and 2004 data*. IFEN.

Jochem, F. J. (1999). Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. *Mar. Biol.* 135, 721–728. doi: 10.1007/s002270050673

Juneau, P., Barnett, A., Méléder, V., Dupuy, C., and Lavaud, J. (2015). Combined effect of high light and high salinity on the regulation of photosynthesis in three diatom species belonging to the main growth forms of intertidal flat inhabiting microphytobenthos. *J. Exp. Mar. Bio. Ecol.* 463, 95–104. doi: 10.1016/j.jembe.2014.11.003

Juneau, P., Qiu, B., and Deblois, C. P. (2007). Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: review. *Toxicol. Environ. Chem.* 89, 609–625. doi: 10.1080/02772240701561569

Jursinic, P., and Stemler, A. (1983). Changes in [C]atrazine binding associated with the oxidation-reduction state of the secondary quinone acceptor of photosystem II. *Plant Physiol.* 73, 703–8. doi: 10.1104/pp.73.3.703

Kirilovsky, D. (2007). Photoprotection in cyanobacteria: the orange carotenoid protein (OCP)-related non-photochemical-quenching mecanism. *Photosynth. Res.* 93, 7–16. doi: 10.1007/s11120-007-9168-y

Knutti, R., Rogelj, J., Sedlacek, J., and Fischer, E. M. (2016). A scientific critique of the two-degree climate change target. *Nat. Geosci* 9, 13–18. doi: 10.1038/ngeo2595

Kolpin, D. W., Thurman, E. M., Lee, E. A., Meyer, M. T., Furlong, E. T., and Glassmeyer, S. T. (2006). Urban contributions of glyphosate and its degradate AMPA to streams in the United States. *Sci. Total Environ.* 354, 191–197. doi: 10.1016/j.scitotenv.2005.01.028

Larras, F., Lambert, A.-S., Pesce, S., Rimet, F., Bouchez, A., and Montuelle, B. (2013). The effect of temperature and a herbicide mixture on freshwater periphytic algae. *Ecotoxicol. Environ. Saf.* 98, 162–170. doi: 10.1016/j.ecoenv.2013.09.007

Legrand, M. F., Costentin, E., and Bruchet, A. (1991). Occurrence of 38 pesticides in various french surface and ground waters. *Environ. Technol.* 12, 985–996. doi: 10.1080/09593339109385097

Li, W.-X., Chen, T.-B., Huang, Z.-C., Lei, M., and Liao, X.-Y. (2006). Effect of arsenic on chloroplast ultrastructure and calcium distribution in arsenic hyperaccumulator *Pteris vittata* L. *Chemosphere* 62, 803–809. doi: 10.1016/j.chemosphere.2005.04.055

Lohrmann, N. L., Logan, B. A., and Johnson, A. S. (2004). Seasonal acclimatization of antioxidants and photosynthesis in *Chondrus crispus* and *Mastocarpus stellatus*, two co-occurring red algae with differing stress tolerances. *Biol. Bull.* 207, 225–232. doi: 10.2307/1543211

Los, D. A., and Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochim. Biophys. Acta Biomembr.* 1666, 142–157. doi: 10.1016/j.bbamem.2004.08.002

MacIntyre, H. L., Kana, T. M., Anning, T., and Geider, R. J. (2002). Review: Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. *J. Phycol.* 38, 17–38. doi: 10.1046/j.1529-8817.2002.00094.x

Mauzerall, D., and Greenbaum, N. L. (1989). The absolute size of a photosynthetic unit. *Biochim. Biophys. Acta* 974, 119–140. doi: 10.1016/S0005-2728(89)80365-2

Maxwell, D. P., Falk, S., and Huner, N. (1995). Growth at low temperature mimics high-light acclimation in Chlorella vulgaris. *Plant Physiol.* 107, 687–684. doi: 10.1104/pp.107.3.687

Mayasich, J. M., Karlander, E., and Terlizzi, J. (1986). Growth responses of Nannochloris oculata Droop and *Phaeodactylum tricornutum* Bohlin to the herbicide atrazine as influenced by light and temperature. *Aquat. Toxicol.* 8, 175–184. doi: 10.1016/0166-445X(86)90063-9

McGinty, E., Pieczonka, J., and Mydlarz, L. (2012). Variations in reactive oxygen release and antioxidant activity in multiple symbiodinium types in response to elevated temperature. *Microb. Ecol.* 64, 1000–1007. doi: 10.1007/s00248-012-0085-z

MDDEP (2008). *Résultats de Cyanobactéries et Cyanotoxines à Sept Stations de Production d'eau Potable* (2004–2006). Quebec Government, Ministère du Développement durable, Environnement et Parcs.

Millie, D. F., Hersh, C. M., and Dionigi, C. P. (1992). Simazine-induced inhibition in photoaclimated populations of *Anabaena circinlis* (cyanophyta). *J. Phycol.* 28, 19–26. doi: 10.1111/j.0022-3646.1992.00019.x

Morgan-Kiss, R., and Dolhi, J. (2012). "Microorganisms and plants: a photosynthetic perspective," in *Temperature Adaptation in a Changing Climate: Nature at Risk*, eds K. Storey and K. Tanino (Cambridge: CABI), 24–44.

Necchi, O. Jr., (2004). Photosynthetic responses to temperature in tropical lotic macroalgae. *Phycol. Res.* 52, 140–148. doi: 10.1111/j.1440-1835.2004.tb00322.x

O'Neal, S. W., and Lembi, C. A. (1983). Effect of simazine on photosynthesis and growth of filamentous algae. *Weed Sci.* 31, 899–903.

Pesce, S., Batisson, I., Bardot, C., Fajon, C., Portelli, C., Montuelle, B., et al. (2009). Response of spring and summer riverine microbial communities following glyphosate exposure. *Ecotoxicol. Environ. Saf.* 72, 1905–1912. doi: 10.1016/j.ecoenv.2009.07.004

Pesce, S., Fajon, C., Bardot, C., Bonnemoy, F., Portelli, C., and Bohatier, J. (2008). Longitudinal changes in microbial planktonic communities of a French river in relation to pesticide and nutrient inputs. *Aquat. Toxicol.* 86, 352–360. doi: 10.1016/j.aquatox.2007.11.016

Pimentel, C., Bernacchi, C., and Long, S. (2007). Limitations to photosynthesis at different temperatures in the leaves of *Citrus limon*. *Brazil. J. Plant Physiol.* 19, 141–147. doi: 10.1590/S1677-04202007000200006

Qiu, H., Geng, J., Ren, H., Xia, X., Wang, X., and Yu, Y. (2013). Physiological and biochemical responses of *Microcystis aeruginosa* to glyphosate and its Roundup® formulation. *J. Hazard. Mater.* 248–249, 172–176. doi: 10.1016/j.jhazmat.2012.12.033

Quednow, K., and Püttmann, W. (2007). Monitoring terbutryn pollution in small rivers of Hesse, Germany. *J. Environ. Monit.* 9, 1337–1343. doi: 10.1039/b711854f

Raven, J. A., and Geider, R. J. (1988). Temperature and algal growth. *New Phytol.* 110, 441–461. doi: 10.1111/j.1469-8137.1988.tb00282.x

Rebich, R. A., Coupe, R. H., and Thurman, E. M. (2004). Herbicide concentrations in the Mississippi River Basin—the importance of chloroacetanilide herbicide degradates. *Sci. Total Environ.* 321, 189–199. doi: 10.1016/j.scitotenv.2003.09.006

Reddy, K. N., Rimando, A. M., and Duke, S. O. (2004). Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. *J. Agric. Food Chem.* 52, 5139–5143. doi: 10.1021/jf049605v

Reupert, R., and Ploeger, E. (1989). Determination of N-herbicides in groundwater, drinking-water and surface water: analytical method and results. *Vom Wasser* 72, 211–233.

Rhee, G. Y., and Gothan, I. J. (1981). The effects of environmental factors on phytoplankton growth: temperature and interactions of temperature with nutrient limitation. *Limnol. Oceanogr.* 26, 635–648. doi: 10.4319/lo.1981.26.4.0635

Romero, D. M., Ríos de Molina, M. C., and Juárez, A. B. (2011). Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*. *Ecotoxicol. Environ. Saf.* 74, 741–747. doi: 10.1016/j.ecoenv.2010.10.034

Ross, J. C., and Vincent, W. F. (1988). Temperature dependence of UV radiation effects on Antartic cyanobacteria. *J. Phycol.* 34, 118–125. doi: 10.1046/j.1529-8817.1998.340118.x

Sandmann, G., Römer, S., and Fraser, P. D. (2006). Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab. Eng.* 8, 291–302. doi: 10.1016/j.ymben.2006.01.005

Sarcina, M., Tobin, M. J., and Mullineaux, C. W. (2001). Diffusion of phycobilisomes on the thylakoid membranes of the cyanobacterium Synechococcus 7942. *J. Biol. Chem.* 276, 46830–46834. doi: 10.1074/jbc.M107111200

Shick, J. M., Romaine-Lioud, S., Ferrier-Pagés, C., and Gattuso, J.-P. (1999). Ultraviolet-B radiation stimulates shikimate pathway-dependent accumulation of mycosporine-like amino acids in the coral Stylophora pistillata despite decreases in its population of symbiotic dinoflagellates. *Limnol. Oceanogr.* 44, 1667–1682. doi: 10.4319/lo.1999.44.7.1667

Siehl, D. (1997). "Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis," in *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*, eds R. Roe, J. Burton, and R. Kuhr (Amsterdam: IOS Press), 37–67.

Sikka, H. C., and Pramer, D. (1968). Physiological effects of fluometuron on some unicellular algae. *Weed Sci.* 16, 296–299.

Smith, R., and Mobley, C. (2008). "Underwater light," in *Photobiology SE - 7*, ed L. Björn (New York, NY: Springer), 131–138.

Sobrino, C., and Neale, P. J. (2007). Short-Term and Long-Term Effects of Temperature on Photosynthesis in the Diatom Thalassiosira Pseudonana Under Uvr Exposures. *J. Phycol.* 43, 426–436. doi: 10.1111/j.1529-8817.2007.00344.x

Sonoike, K., Hihara, Y., and Ikeuchi, M. (2001). Physiological significance of the regulation of photosystem stoichiometry upon high light acclimation of Synechocystis sp. PCC 6803. *Plant Cell Physiol.* 42, 379–384. doi: 10.1093/pcp/pce046

Stachowski-Haberkorn, S., Becker, B., Marie, D., Haberkorn, H., Coroller, L., and de la Broise, D. (2008). Impact of Roundup on the marine microbial community, as shown by an in situ microcosm experiment. *Aquat. Toxicol.* 89, 232–241. doi: 10.1016/j.aquatox.2008.07.004

Staehr, P. A., and Birkeland, M. (2006). Temperature acclimation of growth photosynthesis and respiration in two mesophilic phytoplankton species. *Phycologia* 45, 648–656. doi: 10.2216/06-04.1

Staehr, P. A., and Sand-Jensen, K. A. J. (2006). Seasonal changes in temperature and nutrient control of photosynthesis, respiration and growth of natural phytoplankton communities. *Freshw. Biol.* 51, 249–262. doi: 10.1111/j.1365-2427.2005.01490.x

Steinheimer, T. R. (1993). HPLC determination of atrazine and principal degradates in agricultural soils and associated surface and ground water. *J. Agric. Food Chem.* 41, 588–595. doi: 10.1021/jf00028a016

Sullivan, D. J., Vecchia, A. V., Lorenz, D. L., Gillom, R. J., and Martim, J. D. (2009). *Trends in Pesticide Concentrations in Corn-Belt Streams, 1996–2006*. Reston, VA: National Water-Quality Assessment Program. Scientific Investigations Report 2009-5132. U.S. Department of the Interior and U.S. Geological Survey.

Sunda, W. G., Kieber, D. J., and Kiene, R. P. (2002). An antioxidant function for DMSP and DMS in marine algae. *Nature* 418, 317–320. doi: 10.1038/nature00851

Suzuki, N., and Mittler, R. (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiol. Plant.* 126, 45–51. doi: 10.1111/j.0031-9317.2005.00582.x

Tang, J., Hoagland, K. D., and Siegfried, B. (1998). Uptake and bioconcentration of atrazine by selected freshwater algae. *Environ. Toxicol. Chem.* 17, 1085–1090. doi: 10.1002/etc.5620170614

Tasmin, R., Shimasaki, Y., Qiu, X., Honda, M., Tsuyama, M., Yamada, N., et al. (2014a). Elevated temperatures and low nutrients decrease the toxicity of diuron for growth of the green alga *Pseudokirchneriella subcapitata* subcapitata. *Japan. J. Environ. Toxicol.* 1, 1–10. doi: 10.11403/jset.17.1

Tasmin, R., Shimasaki, Y., Tsuyama, M., Qiu, X., Khalil, F., Okino, N., et al. (2014b). Elevated water temperature reduces the acute toxicity of the widely used herbicide diuron to a green alga, *Pseudokirchneriella subcapitata*. *Environ. Sci. Pollut. Res.* 21, 1064–1070. doi: 10.1007/s11356-013-1989-y

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., et al. (2001). Forecasting agriculturally driven global environmental change. *Science* 292, 281–284. doi: 10.1126/science.1057544

Torres, P. B., Chow, F., Ferreira, M. J. P., and dos Santos, D. Y. A. C. (2016). Mycosporine-like amino acids from *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta) and its variation under high light. *J. Appl. Phycol.* 28, 2035–2040. doi: 10.1007/s10811-015-0708-0

Tsui, M. T. K., and Chu, L. M. (2003). Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere* 52, 1189–1197. doi: 10.1016/S0045-6535(03)00306-0

Tuckey, D. M., Orcutt, D. M., and Hipkins, P. L. L. (2002). Inherent and growth stage-related differences in growth and lipid and sterol composition of algal species sensitive and tolerant to sterol-inhibiting fungicides. *Environ. Toxicol. Chem.* 21, 1715–1723. doi: 10.1002/etc.5620210825

USEPA (2004). *Overview of the Ecological Risk Assessment Process in the Office of Pesticide Programs*. US Environmental Protection Agency. Washington, DC: Office of Prevention, Pesticides and Toxic Substances Office of pesticides Programs.

Vona, V., Di Martino Rigano, V., Lobosco, O., Carfagna, S., Esposito, S., and Rigano, C. (2004). Temperature responses of growth, photosynthesis, respiration and NADH: nitrate reductase in cryophilic and mesophilic algae. *New Phytol.* 163, 325–331. doi: 10.1111/j.1469-8137.2004.01098.x

Wang, C., Lin, X., Li, L., and Lin, S. (2016). Differential growth responses of marine phytoplankton to herbicide glyphosate. *PLoS ONE* 11:e0151633. doi: 10.1371/journal.pone.0151633

Waring, J., Klenell, M., Bechtold, U., Underwood, G. J. C., and Baker, N. R. (2010). Light-induced responses of oxygen photoreduction, reactive oxygen species

production and scavenging in two diatom species. *J. Phycol.* 46, 1206–1217. doi: 10.1111/j.1529-8817.2010.00919.x

Widenfalk, A., Bertilsson, S., Sundh, I., and Goedkoop, W. (2008). Effects of pesticides on community composition and activity of sediment microbes—responses at various levels of microbial community organization. *Environ. Pollut.* 152, 576–584. doi: 10.1016/j.envpol.2007.07.003

Wilkinson, A. D., Collier, C. J., Flores, F., and Negri, A. P. (2015). Acute and additive toxicity of ten photosystem-II herbicides to seagrass. *Sci. Rep.* 5:17443. doi: 10.1038/srep17443

Wilson, K. E., and Huner, N. O. A. (2000). The role of growth rate, redox-state of the plastoquinone pool and the trans-thylakoid ΔpH in photoacclimation of Chlorella vulgaris to growth irradiance and temperature. *Planta* 212, 93–102. doi: 10.1007/s004250000368

Wong, P. K. K. (2000). Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of Scenedesmus quadricauda Berb 614. *Chemosphere* 41, 177–182. doi: 10.1016/S0045-6535(99)00408-7

Wood, R. J., Mitrovic, S. M., Lim, R. P., and Kefford, B. J. (2016). The influence of reduced light intensity on the response of benthic diatoms to herbicide exposure. *Environ. Toxicol. Chem.* 35, 2252–2260. doi: 10.1002/etc.3379

Yan, G., Yan, X., and Wu, W. (1997). Effects of the herbicide molinate on mixotrophic growth, photosynthetic pigments, and protein content of *Anabaena sphaerica* under different light conditions. *Ecotoxicol. Environ. Saf.* 38, 144–149. doi: 10.1006/eesa.1997.1570

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Gomes and Juneau. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

MINI REVIEW
published: 09 December 2016
doi: 10.3389/fenvs.2016.00079

Current Pesticide Risk Assessment Protocols Do Not Adequately Address Differences between Honey Bees (*Apis mellifera*) and Bumble Bees (*Bombus* spp.)

Kimberly A. Stoner *

Department of Entomology, Connecticut Agricultural Experiment Station, New Haven, CT, USA

Recent research has demonstrated colony-level sublethal effects of imidacloprid on bumble bees affecting foraging and food consumption, and thus colony growth and reproduction, at lower pesticide concentrations than for honey bee colonies. However, these studies may not reflect the full effects of neonicotinoids on bumble bees because bumble bee life cycles are different from those of honey bees. Unlike honey bees, bumble bees live in colonies for only a few months each year. Assessing the sublethal effects of systemic insecticides only on the colony level is appropriate for honey bees, but for bumble bees, this approach addresses just part of their annual life cycle. Queens are solitary from the time they leave their home colonies in fall until they produce their first workers the following year. Queens forage for pollen and nectar, and are thus exposed to more risk of direct pesticide exposure than honey bee queens. Almost no research has been done on pesticide exposure to and effects on bumble bee queens. Additional research should focus on critical periods in a bumble bee queen's life which have the greatest nutritional demands, foraging requirements, and potential for exposure to pesticides, particularly the period during and after nest establishment in the spring when the queen must forage for the nutritional needs of her brood and for her own needs while she maintains an elevated body temperature in order to incubate the brood.

Keywords: queen, neonicotinoid, imidacloprid, sublethal effects, nectar consumption, pesticide exposure, incubation

OPEN ACCESS

Edited by:
Johann G. Zaller, University of Natural Resources and Life Sciences, Vienna, Austria

Reviewed by:
Michalis D. Omirou, Agricultural Research Institute, Cyprus
Leif Richardson, The University of Vermont, USA

***Correspondence:**
Kimberly A. Stoner
Kimberly.Stoner@ct.gov

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *19 September 2016*
Accepted: *24 November 2016*
Published: *09 December 2016*

Citation:
Stoner KA (2016) Current Pesticide Risk Assessment Protocols Do Not Adequately Address Differences between Honey Bees (Apis mellifera) and Bumble Bees (Bombus spp.). Front. Environ. Sci. 4:79. doi: 10.3389/fenvs.2016.00079

INTRODUCTION

Bumble bees are major crop pollinators, particularly in temperate ecosystems. Kleijn et al. (2015) ranked 11 North American bumble bee species and seven European species among the 100 top wild bees for crop pollination value worldwide, with *Bombus impatiens, Bombus terrestris/lucorum* (indistinguishable in the field), and *Bombus lapidarius* at the top. Bumble bees pollinate spring-blooming crops and wildflowers under cooler and wetter weather than honey bees (Corbet et al., 1993), and pollinate flowers that require high frequency sonication (King and Buchmann, 2003). Bumble bees are keystone species in natural pollination networks because of the diversity of flowering plants they visit (Memmott et al., 2004), their ability to use flowers requiring complex behavior for pollination (Heinrich, 1979), and the long tongues of some species, allowing them to reach nectar deep in flowers not effectively pollinated by other insects (Corbet, 2000).

The importance of bumble bees to agricultural and natural systems makes the decline in range and abundance of many bumble bee species a matter of great concern (Colla and Packer, 2008; Goulson et al., 2008; Grixti et al., 2009; Williams and Osborne, 2009; Cameron et al., 2011; Colla et al., 2012). Of the 68 species of bumble bees in Europe, 31 (45.6%) are declining (Potts et al., 2015). Surveys of North American bumble bee species found several species in severe decline, regionally or nationally (Grixti et al., 2009; Cameron et al., 2011; Colla et al., 2012; Bartomeus et al., 2013). Similar losses have been found in parts of China (Xie et al., 2008; Williams et al., 2009), Japan (Inoue et al., 2008), and Argentina (Morales et al., 2013).

Multiple factors are implicated for these losses (Goulson et al., 2015) including: loss of long-term flowering habitat (Goulson et al., 2005; Xie et al., 2008; Grixti et al., 2009), increased infection with pathogens and parasites (Cameron et al., 2011, 2016; Szabo et al., 2012; Graystock et al., 2015), displacement of native species by imported commercial species (Inoue et al., 2008; Morales et al., 2013), and climate change (Kerr et al., 2015). The decline in bumble bee abundance and shift from early emerging to later emerging bumble bee species following spring aerial application of fenitrothion to New Brunswick forests shows that pesticide application during this sensitive period can also be a factor (Plowright et al., 1978; Plowright and Rodd, 1980).

Pesticide risk assessments require identifying pesticide concentrations causing adverse effects on species survival and reproduction, understanding the routes and magnitude of pesticide exposure and evaluating these in relation to each other at all stages of the life cycle (Sanchez-Bayo and Tennekes, 2015). Historically, pesticide risk assessments for pollinators focused on acute toxicity, using standardized methods to determine the median lethal dose (LD_{50}) for honey bee workers, and models to quantify honey bee contact exposure from foliar applications (Fischer and Moriarty, 2011). Reviews comparing LD_{50} values among bee species have shown that bumble bee workers are similar to or less sensitive than honey bee workers for most pesticides (Thompson and Hunt, 1999; Thompson, 2001; Mommaerts and Smagghe, 2011; Arena and Sgolastra, 2014; Sanchez-Bayo and Goka, 2014).

With increasing concern about losses of honey bees in North America and Europe (Vanengelsdorp and Meixner, 2010), decline of wild pollinators in Europe (Biesmeijer et al., 2006), and evidence of sublethal effects of pesticides on pollinators (Desneux et al., 2007), assessment of pesticide risks to pollinators has come under increased scrutiny. This scrutiny has particularly focused on neonicotinoid insecticides (Maxim and van der Sluijs, 2010). Although neonicotinoids have been implicated in direct mortality of honey bees (Pistorius et al., 2009; Cutler et al., 2014) and bumble bees (Xerces Society, 2014), the continuing controversy about the risk of neonicotinoid insecticides to bees rests on whether the levels to which bees are exposed cause sublethal effects on the long-term health and survival of bee populations (Blacquière et al., 2012; Godfray et al., 2014, 2015).

DIFFERENCES BETWEEN HONEY BEE AND BUMBLE BEE LIFE CYCLES

Honey bees live in eusocial colonies as superorganisms, with the queen always attended by workers (Straub et al., 2015). A honey bee queen does not forage. She leaves the protective environment of the colony only once, for her mating flight (Sammataro and Avitabile, 1998). Her exposure to pesticides is mediated by workers who collect pollen and nectar and process them into bee bread and honey. Large honey bee colonies can compensate for loss of workers from pesticide exposure, maintaining colony size and honey production, although small colonies may be more susceptible (Henry et al., 2015; Wu-Smart and Spivak, 2016).

In contrast, bumble bees live in colonies for only part of the year, and the potential for the queen to be directly exposed to pesticides, orally and by contact, is much greater than for honey bees. Unlike honey bees, which overwinter as a colony, only the mated bumble bee queen overwinters. In the typical bumble bee life cycle, after overwintering each queen must successfully establish a colony in order to reproduce, although there are also bumble bee species that are inquilines (nest parasites) on other bumble bee species, and individual queens may usurp the nests of other queens (Goulson, 2010). Many bumble bee colonies fail, and many produce only males, with generally only the largest colonies producing both males and new queens (Duchateau and Velthuis, 1988; Müller and Schmid-Hempel, 1992). This may be due to the greater resources required to produce queens. Queen larvae in *Bombus terrestris* require approximately 3X as much food as workers and 2X as much as males (Duchateau and Velthuis, 1988). As a result, males are generally present in excess, although in most species queens mate only once (Goulson, 2010).

The most energy-intensive period of the queen's life is after establishment of a new colony, because she not only has to forage for sufficient pollen and nectar to support herself and her larvae, but also produce enough heat to incubate the developing eggs and larvae (Heinrich, 2004). She heats this brood clump with her own body, maintaining a body temperature of 35–38°C day and night during the first few weeks after nest initiation to keep the brood around 30°C (Heinrich, 1974). Incubating *Bombus vosnesenskii* queens in a laboratory environment (20–23°C), with a 50% sucrose solution supplied so that they did not have to forage, consumed ca. 1 ml. of sucrose solution (0.5 g. of sucrose) per day, 3X as much as non-incubating queens (Heinrich, 1972).

Although for honey bees the gold standard for pesticide risk assessment is to evaluate effects on the long-term survival of the colony as a whole, for bumble bees, the appropriate standard is 2-fold: (1) effects on colony production of new queens and males, and (2) effects on queen success in mating, overwintering, establishing, and supporting a new colony capable of reproducing at the end of the season. A number of studies of sublethal effects of neonicotinoids have addressed foraging, growth, and reproduction of bumble bee colonies (Supplementary Table 1); very few have addressed pesticide exposure to bumble bee queens or possible effects on their fitness.

NEONICOTINOID CONCENTRATIONS WITH SUBLETHAL EFFECTS ON BUMBLE BEE COLONIES

The specifics of 24 studies of the sublethal effects of oral exposure to neonicotinoids in bumble bee colonies are presented in Supplementary Table 1, including whether they were in the laboratory or field, the extent to which foraging was a component, the concentration and duration of exposure, and the species used. One important factor is whether workers traveled for food. This appears most dramatically in Mommaerts et al. (2010). When workers had to travel 20 cm to a separate box for food, exposure to imidacloprid at 10 ppb for 14 days resulted in a significant (60%) loss of reproduction, whereas in an otherwise identical test with the food in the nest box, there was no significant loss of reproduction at 10 or 20 ppb.

Another important distinction is between the studies with queenright colonies (queen plus workers, generally standardized by starting size) and those using micro-colonies (small artificial colonies with 3–5 workers, one of whom becomes dominant and lays eggs). Due to the haplo-diploid system of reproduction in Hymenoptera, unmated workers can produce offspring, but they are always haploid, and thus male (Goulson, 2010). Micro-colonies are used only in laboratory or confined greenhouse experiments (Tasei et al., 2000; Gradish et al., 2010; Mommaerts et al., 2010); queenright colonies are used for field studies, whether the pesticide exposure happens in the field (Larson et al., 2013; Rundlöf et al., 2015), or in the laboratory followed by field foraging (Whitehorn et al., 2012; Feltham et al., 2014), or by simultaneous provision of pesticide-treated sugar water and field foraging (Gill et al., 2012; Gill and Raine, 2014; Moffat et al., 2015, 2016).

Many of the studies in Supplementary Table 1 have demonstrated adverse effects of imidacloprid on *B. terrestris* colonies at concentrations well below the proposed US EPA trigger levels for honey bees (No Observed Adverse Effect Concentration of 25 ppb in nectar and the Lowest Observed Adverse Effect Concentration of 100 ppb in pollen; United States Environmental Protection Agency, 2016). Mommaerts et al. (2010) reported significant loss of reproduction at 10 ppb imidacloprid in micro-colonies required to travel 20 cm for food, and a complete loss of reproduction in queenright colonies at 10 ppb imidacloprid when required to travel 3 m for food. Even without requiring the workers to travel, Laycock et al. (2012) still had a dose-dependent decrease in both sugar water and pollen consumption and a decline in brood production of micro-colonies down to 1.27 ppb imidacloprid with 14 days exposure. In a subsequent laboratory experiment, Laycock and Cresswell (2013) found a dose-dependent reduction in brood production at 0.3–10 ppb of imidacloprid with 14 days of exposure, but the colonies substantially recovered after 14 days off dose. Bryden et al. (2013) measured birth and death rates over time in a laboratory colony at 10 ppb, and found eclosion of new workers was near zero after 21 days.

In studies requiring field foraging with *B. terrestris* and imidacloprid, Whitehorn et al. (2012) found that queenright colonies fed imidacloprid for 14 days at 6 ppb in pollen, and 0.7 ppb in sugar water followed by field foraging had a modest but significant reduction in total colony weight, but a major reduction (85%) in queen production. Feltham et al. (2014) also fed queenright colonies 6 ppb imidacloprid in pollen and 0.7 ppb in sugar water for 14 days and found a subsequent 31% reduction in the rate of field pollen foraging. Gill et al. (2012) and Gill and Raine (2014) used a longer period (28 days) and higher level (10 ppb in sugar water) of exposure, and similarly found effects on the efficiency of field pollen foraging and effects on worker numbers and brood. Moffat et al. (2015), providing colonies foraging freely in the field with a one-time supplement of 1500 ml of sugar water with 2.1 ppb imidacloprid, found significant reductions in colony growth, viable brood and surviving bees at the end of 43 or 48 days. Repeating the experiment the next year at 2.5 ppb, there was again a significant decrease in brood cells, although changes in live bees, nest mass, and number of queens were not significant (Moffat et al., 2016).

In summary, imidacloprid consistently affects foraging and subsequently colony growth and brood production of *B. terrestris* at a level of 10 ppb in sugar water or 6 ppb in pollen and 0.7 ppb in sugar water for an exposure period of 14 days. Even a sugar water supplement to natural foraging at 2.1–2.5 ppb imidacloprid reduced brood production of colonies foraging in the field.

There are fewer studies using *B. impatiens*, and those found colony effects at higher imidacloprid concentrations than for *B. terrestris*. Scholer and Krischik (2014), testing a range of concentrations, found reduced production of males and colony weight at 14 ppb and higher queen mortality at 16 ppb using queenright colonies traveling 30.5 cm for food. Morandin and Winston (2003) found less efficient foraging behavior at 30 ppb in mixed pollen and sugar water patties, but not at 7 ppb, and found no effect of either concentration on colony growth.

Different neonicotinoid compounds have different effects on bumble bees at the levels of neurons, feeding behavior in individual worker bees, and colonies (Kessler et al., 2015; Moffat et al., 2016). Fewer studies have been conducted with thiamethoxam and clothianidin than with imidacloprid. Moffat et al. (2016) directly compared thiamethoxam, clothianidin, and imidacloprid, each provided at 2.5 ppb in a sugar water supplement to naturally foraging colonies in the field, and found that imidacloprid reduced the number of brood cells; thiamethoxam reduced brood cells, live bees, nest mass, and the proportion of females in *B. terretstris*; the only significant effect of clothianidin at that concentration was to *increase* the number of queens produced. By contrast Rundlöf et al. (2015), in a field study with exposure to higher concentrations of clothianidin from the nectar and pollen of seed-treated oilseed rape (mean concentration 13.9 $\pm$ 1.8 ppb in honey bee collected pollen, 5.4 $\pm$ 1.4 ppb in bumble bee nectar), found a 63% reduction in new queens produced, and also significant reductions in colony growth and production of workers and males. Field studies by Cutler and Scott-Dupree (2014) and Sterk et al. (2016) found no effect of clothianidin seed treatment on bumble bee colonies, probably because the concentrations were low—below 0.8 ppb for corn pollen (Cutler and Scott-Dupree, 2014) and from 1.3 ppb to below the level of quantification for oil seed rape pollen (Sterk et al., 2016). In a laboratory study

with *B. impatiens* that also involved a foraging assay, Franklin et al. (2004) found no significant effects on colony health or foraging behavior at 6 ppb and at 36 ppb. In a laboratory micro-colony study, Piiroinen et al. (2016) also found no effect of clothianidin at 1 ppb, except to bees stressed in a behavioral test.

For thiamethoxam, laboratory studies differ on the levels showing negative effects, with Elston et al. (2013) finding significant delays in nest initiation, fewer eggs laid, and zero larvae produced at 10 ppb, while Laycock et al. (2014), testing a range of concentrations, found significant effects on food consumption and oviposition starting only at 39 ppb. Fauser-Misslin et al. (2014), in a long-term laboratory study, found that a mixture of thiamethoxam (4 ppb) and clothianidin (1.5 ppb) resulted in reduced worker survival, reduced production of workers and males, and a 77% reduction in production of new queens. Stanley et al. (2016) found no effect of a 2.4 ppb thiamethoxam sugar water supplement on colony growth, but found effects on field foraging for pollen, with longer foraging bouts producing less pollen for treated bees.

LEVELS OF ORAL EXPOSURE OF BUMBLE BEE COLONIES TO NEONICOTINOIDS IN POLLEN AND NECTAR

There are few direct measurements of pesticides in pollen or nectar collected by bumble bee colonies. David et al. (2016) provides the most comprehensive data for neonicotinoids and fungicides in pollen in rural and urban areas near Sussex (UK). They found thiamethoxam in 100% of their samples of stored bumble bee pollen from rural areas, and at surprisingly high concentrations (mean 18 ppb, median 21 ppb) – higher than in oilseed rape pollen, pollen from wildflowers on the borders of oilseed rape fields, and honey bee pollen from hives adjacent to the oilseed rape fields. No thiamethoxam was found in stored pollen from urban bumble bee nests, but imidacloprid was found in 1/3 of the urban nests at a mean concentration of 6.5 ppb. Rundlöf et al. (2015) measured clothianidin at 5.4 ± 1.4 ppb in bumble bee nectar from colonies adjacent to treated oilseed rape fields with effects described above. These neonicotinoid concentrations are higher than those generally considered "field realistic" in previous reviews (Blacquière et al., 2012; Godfray et al., 2014, 2015), and are also higher than mean or median concentrations in area-wide surveys of trapped honey bee pollen (Chauzat et al., 2006; Stoner and Eitzer, 2013) or stored honey bee bread (Lawrence et al., 2016).

Knowledge Gaps

Pesticide risk assessment requires evaluating toxicity (acute, chronic, and sublethal) and exposure (contact and oral) and then evaluating the relationship between toxicity and exposure (Sanchez-Bayo and Tennekes, 2015). Throughout this complex process, it is crucial to keep the protection goals in mind. A workshop of government, academic, and industry representatives identified the protection goal "to maintain pollinator services and the biodiversity and abundance of bumble bees in a specific area" (Cabrera et al., 2015). These are knowledge gaps I see in achieving this protection goal throughout the bumble bee life cycle:

Effects of Pesticide Exposure of Colonies on Queen Production

The appropriate metric for colony fitness is the production of queens and, to a lesser extent, males, because only mated queens overwinter and establish new colonies. Few of the studies in Supplementary Table 1 reported on queen production (Whitehorn et al., 2012; Larson et al., 2013; Fauser-Misslin et al., 2014; Scholer and Krischik, 2014; Moffat et al., 2016).

TABLE 1 | Critical periods for nutrition of bumble bee queens.

Stage	Typical timing	Typical duration	Food consumption	Species studied	References
Larval development of queens	Late summer	9–10 days	Total over larval development: Pollen = 0.11 g (range 0.75–1.35) in regurgitated mix with nectar and proteins	*B. ruderatus*	Pomeroy, 1979
Initial feeding by new adult queens to prepare for hibernation	Late summer or Fall	3–6 days	Total over 6 days: Pollen = 0.28 g (range 0.22–0.36). Nectar (converted to 50% sugar) = 1.41 g (range 0.98–2.06)	*B. terrestris, B. ruderatus*	Pomeroy, 1979; Přidal and Hofbauer, 1996 (pollen only)
Hibernation	Winter to Early Spring	Variable with climate	Metabolizing fat and glycogen reserves: e.g., *B. lapidarius* consumes 90.6 mg fat (94% of fat reserves and 191 mg dry wt. (75%). Consumption of honey in honey stomach (20% water) Mean = 141 mg (75% of total in stomach)	Mixed species hibernating in Southern England	Alford, 1969
Initial foraging after hibernation to stimulate ovaries	Early Spring	Ca. 18 days	Weight gain of 109 mg over 18 days, consuming both pollen and nectar	*B. lucorum*	Cumber, 1949
Non-incubating queens	Early Spring	3–21 days	0.30–0.38 ml of 50% sucrose per day (laboratory)	*B. vosnesenskii*	Heinrich, 1972
Incubation of brood nest	Late Spring	Ca. 30 days	0.90–1.14 ml of 50% sucrose per day (laboratory)	*B. vosnesenskii*	Heinrich, 1972

Food Consumption of Bumble Bee Queens during Critical Life Stages

Bumble bee queens have several critical periods of foraging between their emergence as adults in the fall and when the first cohort of workers takes over foraging the following spring (**Table 1**). As noted above, spring nest establishment and incubation make the greatest foraging demands on queens. In addition to the energy expenditure for nest incubation (Heinrich, 1972), queens must also expend significant energy in nectar foraging, requiring major investments in warm-up for flight (depending on ambient temperature) and for flight itself. Foraging is required daily, since the queen's energy storage in her honey pot is only sufficient for a few hours of incubation (Heinrich, 2004). Spring foraging is also highly localized to minimize the energy expense of extended flight and minimize time away from the nest (Heinrich, 2004).

The estimates of food consumption for each critical stage in **Table 1** were gleaned from literature on several different species, but at least laboratory estimates for each critical period for *B. terrestris* and *B. impatiens*, which are commercially available and crucial crop pollinators, should be measured. Food consumption at each stage is important to assess acute toxicity by relating pesticide concentration in pollen and nectar to an oral dose per queen bee, which can then be related to the LD_{50} (Stoner and Eitzer, 2013), although both the oral dose and the LD_{50} would need to be standardized for differences in weight between queens and workers and for variation among queens (Thompson and Hunt, 1999).

Major Sources of Nectar and Pollen for Bumble Bee Queens during Critical Life Stages

Detailed studies of pollen and nectar sources of spring bumble bee queens have been made in west Scotland (Brian, 1957), Wisconsin (Macior, 1968), and sub-Alpine environments near Mount Hood in Oregon (Macior, 1994), and could be extracted from season-long surveys of bumble bee visits to flowers (e.g., Fussell and Corbet, 1992) and from analysis of pollen on queens from museum collections (Scheper et al., 2014). Bumble bee queens have been frequently noted as pollinators of spring-blooming fruit trees and bushes (e.g., lowbush blueberry: Stubbs et al., 1992; Javorek et al., 2002; apple: Macior, 1968; Adamson et al., 2012; Russo et al., 2015), and thus use of pesticides on these crops and on spring-blooming ornamental shrubs and trees attractive to bumble bee queens, such as rhododendron, lilac and honeysuckle (Evans et al., 2007), may pose a particular hazard in this critical stage of the bumble bee life cycle.

Potential Effects of Pesticide Exposure on Bumble Bee Queens

There is no data on whether pesticide exposure has sublethal effects on the solitary stages of bumble bee queens. Bumble bee queens have major physiological differences from workers because they build up fat reserves for overwintering, consume those resources during hibernation, and then switch over to ovary development, nest establishment, wax production, and incubation (Votavová et al., 2015). A recent paper (Chaimanee et al., 2016) reported loss of viability of sperm stored in the spermatheca of honey bee queens exposed to 20 ppb of imidacloprid for 7 days. Bumble bee queens mate before overwintering and store sperm for months before beginning oviposition, so they could be similarly affected.

CONCLUSION

Although, eusocial bee colonies are buffered from the effects of pesticides in relation to their size (Henry et al., 2015; Straub et al., 2015; Wu-Smart and Spivak, 2016), and the solitary queen phase of the bumble bee life cycle and early nest establishment are likely the periods of greatest sensitivity to pesticides (Thompson, 2001; Cabrera et al., 2015), current recommendations for additional risk assessment protocols for bumble bees focus exclusively on colonies (Cabrera et al., 2015). These recommendations may become policy not only for neonicotinoids, but also for other pesticides as pollinator health rises in importance to regulatory agencies. There is danger in limiting pesticide risk assessment to what is likely the least sensitive stage of the bumble bee life cycle.

Future pesticide risk assessments should be directed by a model based on expert knowledge of bumble bee ecology throughout the life cycle identifying the most critical points for targeted research (Henry et al., 2016). In the meantime, evaluation of pesticide concentrations that interfere with nest establishment, nectar foraging, and brood incubation, and comparison of those concentrations to probable exposures on pesticide-treated spring blooming crops would be apparent priorities for research.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

FUNDING

Funding for related research came from the US Department of Agriculture National Institute of Food and Agriculture grant NIFA 2011-51181-30673, and from the Connecticut Department of Energy and Environmental Protection.

ACKNOWLEDGMENTS

Thanks to Tracy Zarrillo for invaluable assistance in compiling and summarizing the material in Supplementary Table 1 and to Morgan Lowry for assistance in editing. Thanks also to Bernd Heinrich for the correspondence about nectar consumption during nest incubation that initiated this project, and to the reviewers for helpful comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2016.00079/full#supplementary-material

REFERENCES

Adamson, N. L., Roulston, T. H., Fell, R. D., and Mullins, D. E. (2012). From April to August—Wild bees pollinating crops through the growing season in Virginia, U.*S.A. Environ. Entomol.* 41, 813–821. doi: 10.1603/EN12073

Alford, D. V. (1969). A study of the hibernation of bumblebees (Hymenoptera: Bombidae) in southern England. *J. Anim. Ecol.* 38, 149–170. doi: 10.2307/2743

Arena, M., and Sgolastra, F. (2014). A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology* 23, 324–334. doi: 10.1007/s10646-014-1190-1

Bartomeus, I., Ascher, J. S., Gibbs, J., Danforth, B. N., Wagner, D. L., Hedtke, S. M., et al. (2013). Historical changes in northeastern US bee pollinators related to shared ecological traits. *Proc. Nat. Acad. Sci. U.S.A.* 110, 4656–4660. doi: 10.1073/pnas.1218503110

Biesmeijer, J. C., Roberts, S. P. M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., et al. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* 313, 351–354. doi: 10.1126/science.1127863

Blacquière, T., Smagghe, G., Van Gestel, C. A., and Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* 21, 973–992. doi: 10.1007/s10646-012-0863-x

Brian, A. D. (1957). Differences in the flowers visited by four species of bumble-bees and their causes. *J. Anim. Ecol.* 1, 71–98. doi: 10.2307/1782

Bryden, J., Gill, R. J., Mitton, R. A., Raine, N. E., and Jansen, V. A. (2013). Chronic sublethal stress causes bee colony failure. *Ecol. Lett.* 16, 1463–1469. doi: 10.1111/ele.12188

Cabrera, A. R., Almanza, M. T., Cutler, G. C., Fischer, D. L., Hinarejos, S., Lewis, G., et al. (2015). Initial recommendations for higher-tier risk assessment protocols for bumble bees, *Bombus* spp. (Hymenoptera: Apidae). *Integr. Environ. Assess. Manage.* 12, 222–229. doi: 10.1002/ieam.1675

Cameron, S. A., Lim, H. C., Lozier, J. D., Duennes, M. A., and Thorp, R. (2016). Test of the invasive pathogen hypothesis of bumble bee decline in North America. *Proc. Nat. Acad. Sci. U.S.A.* 113, 4386–4391. doi: 10.1073/pnas.1525266113

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., et al. (2011). Patterns of widespread decline in North American bumble bees. *Proc. Nat. Acad. Sci. U.S.A.* 108, 662–667. doi: 10.1073/pnas.1014743108

Chaimanee, V., Evans, J. D., Chen, Y., Jackson, C., and Pettis, J. S. (2016). Sperm viability and gene expression in honey bee queens (*Apis mellifera*) following exposure to the neonicotinoid insecticide Imidacloprid and the organophosphate Acaricide Coumaphos. *J. Insect Physiol.* 89, 1–8. doi: 10.1016/j.jinsphys.2016.03.004

Chauzat, M. P., Faucon, J. P., Martel, A. C., Lachaize, J., Cougoule, N., and Aubert, M. (2006). A survey of pesticide residues in pollen loads collected by honey bees in France. *J. Econ. Entomol.* 99, 253–262. doi: 10.1093/jee/99.2.253

Colla, S. R., Gadallah, F., Richardson, L., Wagner, D., and Gall, L. (2012). Assessing declines of North American bumble bees (*Bombus* spp.) using museum specimens. *Biodivers. Conserv.* 21, 3585–3595. doi: 10.1007/s10531-012-0383-2

Colla, S. R., and Packer, L. (2008). Evidence for decline in eastern North American bumblebees (Hymenoptera: Apidae), with special focus on *Bombus affinis* Cresson. *Biodivers. Conserv.* 17, 1379–1391. doi: 10.1007/s10531-008-9340-5

Corbet, S. A. (2000). Conserving compartments in pollination webs. *Conserv. Biol.* 14, 1229–1231. doi: 10.1046/j.1523-1739.2000.00014.x

Corbet, S. A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A., et al. (1993). Temperature and the pollinating activity of social bees. *Ecol. Entomol.* 18, 17–30. doi: 10.1111/j.1365-2311.1993.tb01075.x

Cumber, R. A. (1949). The biology of humble bees, with special reference to the production of the worker caste. *Trans. R. Entomol. Soc. Lond.* 100, 1–45. doi: 10.1111/j.1365-2311.1949.tb01420.x

Cutler, G. C., and Scott-Dupree, C. D. (2014). A field study examining the effects of exposure to neonicotinoid seed-treated corn on commercial bumble bee colonies. *Ecotoxicology* 23,1755–1763. doi: 10.1007/s10646-014-1340-5

Cutler, G. C., Scott-Dupree, C. D., and Drexler, D. M. (2014). Honey bees, neonicotinoids, and bee incident reports: the Canadian situation. *Pest Manag. Sci.* 70, 779–783. doi: 10.1002/ps.3613

David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M., et al. (2016). Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environ. Int.* 88, 169–178. doi: 10.1016/j.envint.2015.12.011

Desneux, N., Decourtye, A., and Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* 52, 81–106. doi: 10.1146/annurev.ento.52.110405.091440

Duchateau, M. J., and Velthuis, H. H. W. (1988). Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour* 107, 186–207. doi: 10.1163/156853988X00340

Elston, C., Thompson, H. M., and Walters, K. F. (2013). Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. *Apidologie* 44, 563–574. doi: 10.1007/s13592-013-0206-9

Evans, E., Burns, I., and Spivak, M. (2007). *Befriending Bumble Bees: A Practical Guide to Raising Local Bumble Bees*. Available online at: http://conservancy.umn.edu/handle/11299/51331

Fauser-Misslin, A., Sadd, B. M., Neumann, P., and Sandrock, C. (2014). Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory. *J. Appl. Ecol.* 51, 450–459. doi: 10.1111/1365-2664.12188

Feltham, H., Park, K., and Goulson, D. (2014). Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology* 23, 317–323. doi: 10.1007/s10646-014-1189-7

Fischer, D., and Moriarty, T. (2011). *Pesticide Risk Assessment for Pollinators: Summary of a SETAC Pellston Workshop*. Pensacola, F.L: Society of Environmental Toxicology and Chemistry (SETAC). Available online at: https://c.ymcdn.com/sites/www.setac.org/resource/resmgr/publications_and_resources/executivesummarypollinators.pdf

Franklin, M. T., Winston, M. L., and Morandin, L. A. (2004). Effects of clothianidin on *Bombus impatiens* (Hymenoptera: Apidae) colony health and foraging ability. *J. Econ. Entomol.* 97, 369–373. doi: 10.1603/0022-0493-97.2.369

Fussell, M., and Corbet, S. A. (1992). Flower usage by bumble-bees: a basis for forage plant management. *J. Appl. Ecol.* 29, 451–465. doi: 10.2307/2404513

Gill, R. J., and Raine, N. E. (2014). Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Funct. Ecol.* 28, 1459–1471. doi: 10.1111/1365-2435.12292

Gill, R. J., Ramos-Rodriguez, O., and Raine, N. E. (2012). Combined pesticide exposure severely affects individual-and colony-level traits in bees. *Nature* 491, 105–108. doi: 10.1038/nature11585

Godfray, H. C. J., Blacquière, T., Field, L. M., Hails, R. S., Petrokofsky, G., Potts, S. G., et al. (2014). A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. Biol. Sci.* 281:20140558. doi: 10.1098/rspb.2014.0558

Godfray, H. C., Blacquiere, T., Field, L. M., Hails, R. S., Potts, S. G., Raine, N. E., et al. (2015). A restatement of recent advances in the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. Biol. Sci.* 282:20151821. doi: 10.1098/rspb.2015.1821

Goulson, D. (2010). *Bumblebees: Behaviour, Ecology, and Conservation, 2nd Edn*. Oxford: Oxford University Press.

Goulson, D., Hanley, M. E., Darvill, B., Ellis, J. S., and Knight, M. E. (2005). Causes of rarity in bumblebees. *Biol. Conserv.* 122, 1–8. doi: 10.1016/j.biocon.2004.06.017

Goulson, D., Lye, G. C., and Darvill, B. (2008). Decline and conservation of bumble bees. *Annu Rev. Entomol.* 53, 191–208. doi: 10.1146/annurev.ento.53.103106.093454

Goulson, D., Nicholls, E., Botías, C., and Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347:1255957. doi: 10.1126/science.1255957

Gradish, A. E., Scott-Dupree, C. D., Shipp, L., Harris, C. R., and Ferguson, G. (2010). Effect of reduced risk pesticides for use in greenhouse vegetable production on *Bombus impatiens* (Hymenoptera: Apidae). *Pest Manag. Sci.* 66, 142–146. doi: 10.1002/ps.1846

Graystock, P., Blane, E. J., McFrederick, Q. S., Goulson, D., and Hughes, W. O. (2015). Do managed bees drive parasite spread and emergence in wild bees? *Int. J. Parasitol. 5*, 64–75. doi: 10.1016/j.ijppaw.2015.10.001

Grixti, J. C., Wong, L. T., Cameron, S. A., and Favret, C. (2009). Decline of bumble bees (*Bombus*) in the North American Midwest. *Biol. Conserv.* 142, 75–84. doi: 10.1016/j.biocon.2008.09.027

Heinrich, B. (1972). Physiology of brood incubation in the bumblebee queen, *Bombus vosnesenskii. Nature* 239, 223–225. doi: 10.1038/239223a0

Heinrich, B. (1974). Thermoregulation in bumblebees. I. Brood incubation by *Bombus vosnesenskii* queens. *J. Comp. Physiol.* 88, 129–140. doi: 10.1007/BF00695404

Heinrich, B. (1979). "Majoring" and "minoring" by foraging bumblebees, *Bombus vagans*: an experimental analysis. *Ecology* 60, 245–255. doi: 10.2307/1937652

Heinrich, B. (2004). *Bumblebee Economic, 2nd Edn*. Cambridge, MA: Harvard University Press.

Henry, M., Becher, M. A., Osborne, J. L., Kennedy, P. J., Aupinel, P., Bretagnolle, V., et al. (2016). Predictive systems models can help elucidate bee declines driven by multiple combined stressors. *Apidologie*. doi: 10.1007/s13592-016-0476-0. [Epub ahead of print].

Henry, M., Cerrutti, N., Aupinel, P., Decourtye, A., Gayrard, M., Odoux, J. F., et al. (2015). Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees. *Proc. R. Soc. B* 282:20152110. doi: 10.1098/rspb.2015.2110

Inoue, M. N., Yokoyama, J., and Washitani, I. (2008). Displacement of Japanese native bumblebees by the recently introduced *Bombus terrestris* (L.)(*Hymenoptera: Apidae*). *J. Insect Conserv.* 12, 135–146. doi: 10.1007/s10841-007-9071-z

Javorek, S. K., Mackenzie, K. E., and Vander Kloet, S. P. (2002). Comparative pollination effectiveness among bees (Hymenoptera: Apoidea) on lowbush blueberry (Ericaceae: Vaccinium angustifolium). *Ann. Entomol. Soc. Am.* 95, 345–351. doi: 10.1603/0013-8746(2002)095[0345:CPEABH]2.0.CO;2

Kerr, J. T., Pindar, A., Galpern, P., Packer, L., Potts, S. G., Roberts, S. M., et al. (2015). Climate change impacts on bumblebees converge across continents. *Science* 349, 177–180. doi: 10.1126/science.aaa7031

Kessler, S. C., Tiedeken, E. J., Simcock, K. L., Derveau, S., Mitchell, J., Softley, S., et al. (2015). Bees prefer foods containing neonicotinoid pesticides. *Nature* 521, 74–76. doi: 10.1038/nature14414

King, M. J., and S. L., Buchmann (2003). Floral sonication by bees: Mesosomal vibration by *Bombus* and *Xylocopa*, but not *Apis* (Hymenoptera: Apidae), ejects pollen from poricidal anthers. *J. Kansas Entomol. Soc.* 76, 295–305. Available online at: http://www.jstor.org/stable/i25086090

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., et al. (2015). Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nat. Commun.* 6, 7414. doi: 10.1038/ncomms8414

Larson, J. L., Redmond, C. T. and Potter, D. A. (2013). Assessing insecticide hazard to bumble bees foraging on flowering weeds in treated lawns. *PLoS ONE* 8:e66375. doi: 10.1371/journal.pone.0066375

Lawrence, T. J., Culbert, E. M., Felsot, A. S., Hebert, V. R., and Sheppard, W. S. (2016). Survey and risk assessment of *Apis mellifera* (Hymenoptera: Apidae) exposure to neonicotinoid pesticides in urban, rural, and agricultural settings. *J. Econ. Entomol.* 109, 520–528. doi: 10.1093/jee/tov397

Laycock, I., Cotterell, K. C., O'Shea-Wheller, T. A., and Cresswell, J. E. (2014). Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees. *Ecotoxicol. Environ. Saf.* 100, 153–158. doi: 10.1016/j.ecoenv.2013.10.027

Laycock, I., and Cresswell, J. E. (2013). Repression and recuperation of brood production in *Bombus terrestris* bumble bees exposed to a pulse of the neonicotinoid pesticide imidacloprid. *PLoS ONE* 8:e79872. doi: 10.1371/journal.pone.0079872

Laycock, I., Lenthall, K. M., Barratt, A. T., and Cresswell, J. E. (2012). Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*). *Ecotoxicology* 21, 1937–1945. doi: 10.1007/s10646-012-0927-y

Macior, L. W. (1968). *Bombus* (Hymenoptera, Apidae) queen foraging in relation to vernal pollination in Wisconsin. *Ecology* 49, 20–25. doi: 10.2307/1933556

Macior, L. W. (1994). Pollen-foraging dynamics of subalpine bumblebees (*Bombus* Latr.). *Plant Species Biol.* 9, 99–106. doi: 10.1111/j.1442-1984.1994.tb00089.x

Maxim, L., and van der Sluijs, J. P. (2010). Expert explanations of honeybee losses in areas of extensive agriculture in France: Gaucho® compared with other supposed causal factors. *Environ. Res. Lett.* 5:014006. doi: 10.1088/1748-9326/5/1/014006

Memmott, J., Waser, N. M., and Price, M. V. (2004). Tolerance of pollination networks to species extinctions. *Proc. R. Soc. B.* 271, 2605–2611. doi: 10.1098/rspb.2004.2909

Moffat, C., Buckland, S. T., Samson, A. J., McArthur, R., Pino, V. C., Bollan, K. A., et al. (2016). Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees. *Sci. Rep.* 6:24764. doi: 10.1038/srep24764

Moffat, C., Pacheco, J. G., Sharp, S., Samson, A. J., Bollan, K. A., Huang, J., et al. (2015). Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). *FASEB J.* 29, 2112–2119. doi: 10.1096/fj.14-267179

Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G., and Smagghe, G. (2010). Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. *Ecotoxicology* 19, 207–215. doi: 10.1007/s10646-009-0406-2

Mommaerts, V., and Smagghe, G. (2011). "Side-effects of pesticides on the pollinator *Bombus*: an overview," in *Pesticides in the Modern World–Pests Control and Pesticides Exposure and Toxicity Assessment,* ed M. Stoytcheva (Rijeka: InTech), 508–552.

Morales, C. L., Arbetman, M. P., Cameron, S. A., and Aizen, M. A. (2013). Rapid ecological replacement of a native bumble bee by invasive species. *Front. Ecol. Environ.* 11:529–534. doi: 10.1890/120321

Morandin, L. A., and Winston, M. L. (2003). Effects of novel pesticides on bumble bee (Hymenoptera: Apidae) colony health and foraging ability. *Environ. Entomol.* 32, 555–563. doi: 10.1603/0046-225X-32.3.555

Müller, C. B., and Schmid-Hempel, P. (1992). Correlates of reproductive success among field colonies of *Bombus lucorum*: the importance of growth and parasites. *Ecol. Entomol.* 17, 343–353. doi: 10.1111/j.1365-2311.1992.tb01068.x

Piiroinen, S., Botías, C., Nicholls, E., and Goulson, D. (2016). No effect of low-level chronic neonicotinoid exposure on bumblebee learning and fecundity. *PeerJ* 4:e1808. doi: 10.7717/peerj.1808

Pistorius, J., Bischoff, G., Heimbach, U., and Stähler, M. (2009). Bee poisoning incidents in Germany in spring 2008 caused by abrasion of active substance from treated seeds during sowing of maize. *Julius Kühn Archiv.* 423, 118–126. Available online at: http://fera.co.uk/news/resources/documents/chem-JKI_Archiv_423.pdf

Plowright, R. C., Pendrel, B. A., and McLaren, I. A. (1978). The impact of aerial fenitrothion spraying upon the population biology of bumble bees (*Bombus* Latr.: Hym.) in south-western New Brunswick. *Can. Entomol.* 110, 1145–1156.

Plowright, R. C., and Rodd, F. H. (1980). The effect of aerial insecticide spraying on hymenopterous pollinators in New Brunswick. *Can. Entomol.* 112, 259–269. doi: 10.4039/Ent112259-3

Pomeroy, N. (1979). Brood bionomics of *Bombus ruderatus* in New Zealand (Hymenoptera: Apidae). *Can. Entomol.* 111, 865–874. doi: 10.4039/Ent111865-8

Potts, S., Biesmeijer, K., Bommarco, R., Breeze, T., Carvalheiro, L., and Franzen, M. (2015). *Status and Trends of European pollinators. Key Findings of the STEP project*. Sofia: Pensoft Publishers. Available online at: http://step-project.net/img/uplf/STEP%20brochure%20online-1.pdf

Přidal, A., and Hofbauer, J. (1996). Laboratory rearing and nutrition of young queens of bumblebee (*Bombus terrestris* L.) from emergence to diapause. *Sci. Stud. Res. Inst. Fodder Plants Troubsko* 14, 125–131.

Rundlöf, M., Andersson, G. K., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., et al. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* 521, 77–80. doi: 10.1038/nature14420

Russo, L., Park, M., Gibbs, J., and Danforth, B. (2015). The challenge of accurately documenting bee species richness in agroecosystems: bee diversity in eastern apple orchards. *Ecol. Evol.* 5, 3531–3540. doi: 10.1002/ece3.158

Sammataro, D., and Avitabile, A. (1998). *The Beekeeper's Handbook, 3rd Edn*. Ithaca, NY: Cornell University Press.

Sanchez-Bayo, F., and Goka, K. (2014). Pesticide residues and bees–a risk assessment. *PLoS ONE* 9:e94482. doi: 10.1371/journal.pone.0094482

Sánchez-Bayo, F., and Tennekes, H. A. (2015). *Environmental Risk Assessment of Agrochemicals — A Critical Appraisal of Current Approaches, Toxicity and Hazard of Agrochemicals,* ed M. Larramendy (InTech). Available online at: http://www.intechopen.com/books/toxicity-and-hazard-of-agrochemicals/environmental-risk-assessment-of-agrochemicals-a-critical-appraisal-of-current-approaches

Scheper, J., Reemer, M., van Kats, R., Ozinga, W. A., van der Linden, G. T., Schaminée, J. H., et al. (2014). Museum specimens reveal loss of pollen host

plants as key factor driving wild bee decline in The Netherlands. *Proc. Natl. Acad. Sci. U.S.A.* 111, 17552–17557. doi: 10.1073/pnas.1412973111

Scholer, J., and Krischik, V. (2014). Chronic exposure of imidacloprid and clothianidin reduce queen survival, foraging, and nectar storing in colonies of *Bombus impatiens*. *PLoS ONE* 9:e91573. doi: 10.1371/journal.pone.0091573

Stanley, D. A., Russell, A. L., Morrison, S. J., Rogers, C., and Raine, N. E. (2016). Investigating the impacts of field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *J. Appl. Ecol.* 53, 1440–1440. doi: 10.1111/1365-2664.12689

Sterk, G., Peters, B., Gao, Z., and Zumkier, U. (2016). Large-scale monitoring of effects of clothianidin-dressed OSR seeds on pollinating insects in Northern Germany: effects on large earth bumble bees (*Bombus terrestris*). *Ecotoxicology* 25, 1–13. doi: 10.1007/s10646-016-1723-x

Stoner, K. A., and Eitzer, B. D. (2013). Using a hazard quotient to evaluate pesticide residues detected in pollen trapped from honey bees (*Apis mellifera*) in Connecticut. *PLoS ONE* 8:e77550. doi: 10.1371/journal.pone.0077550

Straub, L., Williams, G. R., Pettis, J., Fries, I., and Neumann, P. (2015). Superorganism resilience: eusociality and susceptibility of ecosystem service providing insects to stressors. *Curr. Opin. Insect Sci.* 12, 109–112. doi: 10.1016/j.cois.2015.10.010

Stubbs, C. S., Jacobson, H. A., Osgood, E. A., and Drummond, F. A. (1992). *Alternative Forage Plants for Native (Wild) Bees Associated with Lowbush Blueberry, Vaccinium spp., in Maine* (No. 148). Orono, ME: University of Maine.

Szabo, N. D., Colla, S. R., Wagner, D. L., Gall, L. F., and Kerr, J. T. (2012). Do pathogen spillover, pesticide use, or habitat loss explain recent North American bumblebee declines? *Conserv. Lett.* 5, 232–239. doi: 10.1111/j.1755-263X.2012.00234.x

Tasei, J. N., Lerin, J., and Ripault, G. (2000). Sub-lethal effects of imidacloprid on bumblebees, *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. *Pest Manage. Sci.* 56, 784–788. doi: 10.1002/1526-4998(200009)56:9andlt;784::AID-PS208andgt;3.0.CO;2-T

Thompson, H. M. (2001). Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.). *Apidologie* 32, 305–321. doi: 10.1051/apido:2001131

Thompson, H. M., and Hunt, L. V. (1999). Extrapolating from honeybees to bumblebees in pesticide risk assessment. *Ecotoxicology* 8, 147–166. doi: 10.1023/A:1026444029579

USEPA (United States Environmental Protection Agency) (2016). *Preliminary Pollinator Assessment to Support the Registration Review of Imidacloprid. EPA-HQ-OPP-2008-0844-0140*. Available online at: https://www.regulations.gov/document?D=EPA-HQ-OPP-2008-0844-0140

Vanengelsdorp, D., and Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J. Invertebr. Pathol.* 103, S80–S95. doi: 10.1016/j.jip.2009.06.011

Votavová, A., Tomčala, A., Kofroňová, E., Kudzejová, M., Šobotník, J., Jiroš, P., et al. (2015). Seasonal dynamics in the chemistry and structure of the fat bodies of bumblebee queens. *PLoS ONE* 10:e0142261. doi: 10.1371/journal.pone.0142261

Whitehorn, P. R., O'Connor, S., Wackers, F. L., and Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* 336, 351–352. doi: 10.1126/science.1215025

Williams, P., Colla, S., and Xie, Z. (2009). Bumblebee vulnerability: common correlates of winners and losers across three continents. *Conserv. Biol.* 23, 931–940. doi: 10.1111/j.1523-1739.2009.01176.x

Williams, P. H., and Osborne, J. L. (2009). Bumblebee vulnerability and conservation world-wide. *Apidologie* 40, 367–387. doi: 10.1051/apido/2009025

Wu-Smart, J., and Spivak, M. (2016). Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development. *Sci. Rep.* 6:32108. doi: 10.1038/srep32108

Xerces Society (2014). *The Wilsonville Bee Kill*. Available online at: http://www.xerces.org/the-wilsonville-bee-kill/

Xie, Z., Williams, P. H., and Tang, Y. (2008). The effect of grazing on bumblebees in the high rangelands of the eastern Tibetan Plateau of Sichuan. *J. Insect Conserv.* 12, 695–703. doi: 10.1007/s10841-008-9180-3

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Stoner. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

MINI REVIEW
published: 26 June 2017
doi: 10.3389/fenvs.2017.00032

Systematic Review of the Effects of Chemical Insecticides on Four Common Butterfly Families

Rosaria Mulé[1], *Giorgio Sabella*[1], *Lavinia Robba*[2] *and Barbara Manachini*[1]*

[1] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy, [2] Consultant, Palermo, Italy

Safeguarding crop productivity by protecting crops from pest attacks entails the wide use of plant protection products that provide a quick, easy and cheap solution. The objective of this study is to understand the effects of insecticides used in agriculture on non-target butterflies, specifically on the families Lycaenidae, Nymphalidae, Hesperiidae, and Papilionidae. To achieve this goal, a formal systematic review was performed according to European Food Safety Authority (EFSA) guidelines, by entering a combination of keywords on 3 online databases. Three reviewers independently extracted information on study characteristics and quality. The main results were collected and grouped by the insecticide used, butterflies species and family, and endpoints. The output was valuable but heterogeneous as the endpoints and methodologies of the studies reviewed were different. Few experimental studies on the effects of insecticides on the most common butterfly families have been published. Naled and permethrin are the most commonly used insecticides in the experiments, whilst the target organisms of these studies are *Vanessa cardui*, *Danaus plexippus*, *Heliconius charitonius*, belonging to the Nymphalidae family, and *Eumaeus atala*, belonging to the Lycaenidae family; the effects were evaluated on all developmental stages, with special attention to the larval phase. This systematic review highlights the need for more studies on the effects of chemical insecticides on non-target Lepidoptera in light of their ecological importance and the extensive use of these chemical products.

Keywords: Lepidoptera, plant protection products, non-target, risk assessment, pesticides

OPEN ACCESS

Edited by:
Johann G. Zaller, University of Natural Resources and Life Sciences, Vienna, Austria

Reviewed by:
Leif Abrell, University of Arizona, United States
Nemat O. Keyhani, University of Florida, United States

***Correspondence:**
Barbara Manachini
barbara.manachini@unipa.it

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *21 November 2016*
Accepted: *01 June 2017*
Published: *26 June 2017*

Citation:
Mulé R, Sabella G, Robba L and Manachini B (2017) Systematic Review of the Effects of Chemical Insecticides on Four Common Butterfly Families. Front. Environ. Sci. 5:32. doi: 10.3389/fenvs.2017.00032

INTRODUCTION

Agriculture is the most common form of land use in Europe. Modern agricultural lands are often subject to intensified use, characterized by increased field sizes, decreased crop diversity and reduced availability of semi-natural habitats. Moreover, they are subject to high inputs of agrochemicals, mainly Plant Protection Products (PPPs) (Hahn et al., 2015), used to safeguard agricultural production from pests (Sciarra et al., 2015). Globally, agricultural producers apply approximately 3 million tons of pesticides per annum, worth around USD 40 billion (Popp et al., 2012); insecticide use reached 12.2 billion in 2015, and the market is projected to reach more than16.4 billion by 2019 (AAVV, 2015). Insecticides are widely used to control insect pests, but a number of concerns have arisen regarding their environmental safety.

Insecticides are found almost everywhere, and this contamination puts the environment and non-target organisms, ranging from beneficial soil microorganisms, to insects, fishes and birds, at increased risk (Aktar et al., 2009). The use of insecticides in agriculture is well documented as one cause of pollinator declines, especially when spraying times coincide with flowering times (Nicholls and Altieri, 2013). Several studies have suggested that butterflies are key taxa and good indicators for the monitoring of anthropogenic disturbance, including the effect of xenobiotics, and habitat quality (Bonebrake et al., 2010; EEA, 2013). In addition, it has been estimated that approximately 70% of butterfly species (Papilionidae and Hesperiidae) occur in arable land (Boriani et al., 2005; Fileccia et al., 2015), potentially exposing them to various insecticide intensities, depending on their spatial and temporal overlap with applications.

Butterfly populations, both larvae and adults, are at risk of exposure to single and multiple insecticide applications coming from direct spraying or indirect residual deposits on plant tissue, especially as larval periods can coincide with the timing of insecticide applications (Hoang et al., 2011). Considering the enormous quantity of insecticides applied in agriculture, the importance of butterflies as bioindicators and the global decline of several butterflies species, understanding the impact of insecticides on this taxa has become paramount.

The objective of this study was to follow European Food Safety Authority (EFSA) guidelines (EFSA, 2010) in order to carry out a systematic review of published studies to gauge the extent of current knowledge regarding the effects of agricultural insecticide use on non-target butterflies. There are a number of reasons for choosing butterflies as a case study. Butterflies are certainly among the most popular insects for their attractive appearance, and many species play important roles in the ecosystem as pollinators of many wild and cultivated plants. They are also key taxa for biodiversity monitoring because they reflect changes in climatic conditions as well as seasonal and other ecological changes (Fileccia et al., 2015). In addition, butterflies are small, have high reproductive rates and a low trophic level which allows them to quickly respond to environmental stress (Griffis et al., 2001). In this study, we specifically focus our attention on the Lycaenidae, Nymphalidae, Hesperiidae, and Papilionidae, chosen for their sensitivity to stress and presence in a large number of habitats, especially agro-ecosystems.

Lycaenidae is the second-largest family of butterflies (Fiedler, 1996). The majority of lycaenids have associations with ants, which can be facultative or obligate and range from mutualism to parasitism (Pierce et al., 2002). Nymphalidae is the largest family of butterflies (Fiedler, 1998) and includes popular species such as the Monarch butterfly, which has received a lot of attention because it is a migratory, charismatic species (Gullan and Cranston, 2008). The Hesperiidae family, commonly known as "skipper butterflies," are recognized by their quick, darting flight habits (Wang et al., 2013). Finally, Papilionidae includes some of the most spectacular and magnificent of all insects (Collins and Morris, 1985), and they are recognized as model organisms in ecology, evolutionary biology, genetics, and conservation biology (Zakharov et al., 2004).

MATERIALS AND METHODS

Search Criteria

This systematic review was performed following the steps of the EFSA guidelines (EFSA, 2010) as closely as possible. Two research questions were asked: (1) Do agricultural insecticides cause negative effects on non-target butterflies?; and (2) If so, what are they? To answer these questions, systematic research of the available literature in three databases [Scopus (www.scopus.com), Summon (www.unipa.it/amministrazione/area1/ssp04/set11/summon/) and Web of Science (https://apps.webofknowledge.com)] was conducted, with a combination of the following keywords: "Lepidoptera," "butterfly," "butterflies," "non-target," "Lycaenidae," "Nymphalidae," "Hesperiidae," "Papilionidae," "Danaidae," which were combined with "insecticides," "pesticides," and "plant protection product." Searches were conducted in English and Italian on literature from between 1970 and (15 Jan) 2016.

Screening of Search Results

Duplicates were removed manually and abstracts were screened by two screeners against the target research questions. Exclusion criteria described below were developed and selected. Disagreements between the two screeners were resolved by a third screener when necessary. Cross-checking was performed on the excluded articles. Full-text review was independently conducted by three reviewers and reasons for exclusion were annotated and tracked (e.g., "review paper with no original data"). The primary reasons for excluding papers were: (i) articles completely un-related to search questions (biological, ecological, etc.); (ii) general knowledge papers; and (iii) papers that did not follow the basic criteria of scientific research (e.g., replication, minimum in laboratory and/or field standards). Articles clearly meeting the inclusion criteria were obtained for full-text review unless unavailable. These included articles related to the search inquiry, providing that scientific laboratory experiments or field studies had a minimum number of replicas and a negative control. Articles that could not be assessed for relevance based on the title and abstract screening were also subjected to full-text review. Articles were not considered further when their title and abstract clearly indicated that the study did not meet the inclusion criteria.

Data Analyses

Considering the heterogeneity of the data, observations of the effect of treatment compared to the control were extracted for all investigations, and for both laboratory and field studies. The mean of the effect and confidence intervals were used to calculate effect size lr as follows: $lr = \ln(MH/MC)$, where MC is the mean effect, considering the natural mortality recorded, on the control group, and MH the mean effect on the exposed group.

RESULTS AND DISCUSSION

Search outputs for generic keywords such as "Lepidoptera," "butterfly," and "non-target" in three databases were very

extensive, and a total of 192,268 studies were found. Keywords such as "Lepidoptera" or "butterfly" include harmful phytofagous species, so a second search focusing on the most prevalent families of non-target butterflies was conducted. A total of 2097 scientific articles were recorded and then read. After deleting duplicates, we selected 7 articles (**Table 1**) that were useful to answering the proposed research questions.

The review shows that 4 studies were laboratory experiments and 3 were field studies. Overall, 6 different insecticides were tested, and the most common were naled and permethrin. The first is an organophosphate insecticide initially registered for use against adult mosquitoes, but which is also used in agriculture, especially in the United States on cotton crops and alfalfa. The second is a synthetic pyrethroid which, acting as a neurotoxin, affects the nervous system of the organisms and is used in agriculture on cotton, maize and wheat crops. The other insecticides considered in the experiments were dichlorvos, resmethrin, malathion, and imidacloprid. The latter is the second most used PPP in the world, though since 2013 it has been banned in Europe on crops that attract bees because it is considered highly toxic for them (Goulson, 2013). In addition, the U.S. Environmental Protection Agency classifies naled, dichlorvos, and permethrin as highly toxic to aquatic organisms and honeybees, based on acute toxicity data (Hoang et al., 2011).

The Nymphalidae and Lycaenidae have been studied more than Hesperiidae and Papilionidae. The effects of insecticides have been assessed on a total of 20 species, 5 belonging to the Hesperiidae family, 5 to Lycaenidae, 8 to Nymphalidae, and 2 to Papilionidae. The most studied species were *Vanessa cardui*, *Danaus plexippus,* and *Heliconius charitonius*, belonging to Nymphalidae, and *Eumaeus atala*, belonging to Lycaenidae. In addition, 11 species were studied in the field, 1 in the laboratory

TABLE 1 | Studies reviewed in detail in which experiments were carried out to evaluate the effects of insecticides (In) (D, dichlorvos; I, imidacloprid; M, malathion; N, naled; P, permethrin; R, resmethrin) on non-target butterflies.

Study	Experiment	In	Exposure modality	Endpoint	Family	Species	Life stage
Hoang et al., 2011	Laboratory	P N D	Direct applications on thorax and wings	LD_{50} (μg/g), Mortality	Lycaenidae Nymphalidae Nymphalidae Nymphalidae Nymphalidae	*Eumaeus atala* *Heliconius charitonius* *Junonia coenia* *Vanessa cardui* *Anartia jatrophae*	Larva Adult
Zhong et al., 2010	Field	N	Aerial application	Mortality	Lycaenidae	*Cyclargus thomasi bethune bakeri*	Larva
Oberhauser et al., 2009	Field	R	Aerial application	Mortality	Nymphalidae	*Danaus plexippus*	Larva
Bargar, 2012	Field	N	Spray application	Mortality Risk assessment	Papilionidae Papilionidae Nymphalidae Nymphalidae Nymphalidae Nymphalidae Nymphalidae Nymphalidae Nymphalidae Lycaenidae Lycaenidae Lycaenidae Lycaenidae Hesperiidae Hesperiidae Hesperiidae Hesperiidae Hesperiidae	*Papilio polyxenes* *Papilio troilus* *Vanessa cardui* *Junonia coenia* *Agraulis vanilla* *Anartia jatrophae* *Heliconius charitonius* *Phyciodes phaon* *Hermeuptychia sosybius* *Leptotes cassius* *Strymon istapa* *Eumaeus atala* *Calycopis cecrops* *Urbanus proteus* *Erynnis* spp. *Thorybes* spp. *Pyrgus* sp. *Pyrgus oileus*	Adult
Oberhauser et al., 2006	Laboratory	P	Oral administration	Mortality survival, feeding interruption, female oviposition choice	Nymphalidae	*Danaus plexippus*	Larva Adult
Krischik et al., 2015	Laboratory	I	Oral administration	Survival, feeding interruption, fecundity, hatching	Nymphalidae Nymphalidae	*Danaus plexippus* *Vanessa cardui*	Larva Adult
Salvato, 2001	Laboratory	N M P	Direct applications on thorax	LD_{50} (μg/g)	Hesperiidae Hesperiidae Lycaenidae Nymphalidae Nymphalidae	*Urbanus proteus* *Pyrgus oileus* *Eumaeus atala* *Agraulis vanilla* *Heliconius charitonius*	Larva Adult

and 8 in both. Regarding the vital stage, experiments were carried out at the larval stage in 6 of 7 studies, the adult stage in 5 of 7 studies, and at both adult and larval stages in 4 of 7 studies. Moreover, the experiments included 10 species at the adult stage, 1 species at the larval stage and 9 at both. The reported data in Hoang et al. (2011), for example, showed that the fifth larval stage was slightly more sensitive to the tested insecticides compared to the adult stage.

A real meta-analysis was impossible to carry out because the reported data in the selected studies, albeit valid, often differed among themselves. Whilst some studies were conducted in the laboratory, others were in the field, with different exposure modalities. For example, some experiments used oral insecticide administration, whilst others employed direct contact on the thorax or wings. In addition, the endpoints examined often differed among studies because some experiments studied the lethal dosage and others the percentage of mortality or feeding behavior.

However, all insecticides had a negative effect on all species for both stages. **Figure 1** reports the effect size (*lr*) of the six insecticides on larvae and adults. Insecticides have more negative effects on larvae than on adults, except for some specific species. The lowest effects was observed for larvae of *V. cardui* exposed to naled and for *Junonia coenia* exposed to dichlorvos; the most dramatic effect was observed for *E. atala* and *H. charitonius* exposed to permethrin.

CONCLUSION

This review shows that the use of insecticides reported in **Table 1** cause negative effects on the most common butterfly families, such as reduced survival rate, feeding interruption, and alteration of oviposition behavior. However, despite the billions of dollars spent on insecticides and the known importance of Lepidoptera, it has been impossible to determine which species is the most sensitive or which insecticide is the most toxic toward the studied species, given the small number of published studies, different methodological approaches and different endpoints examined. Even though it was not possible to perform an exhaustive meta-analysis given the heterogeneity of data and methodological approaches, it is clear, from this review, that the different species have different susceptibility to different insecticides. However, as this review manuscript aims to assist in the formulation of policies, by offering sound scientific evidence on the effects of PPPs on non-target organisms (in this case, butterflies) that are recognized worldwide as good indicators, it is important to mention the lack of data encountered on the concept of sub-lethal effects. This highlights the need for further research

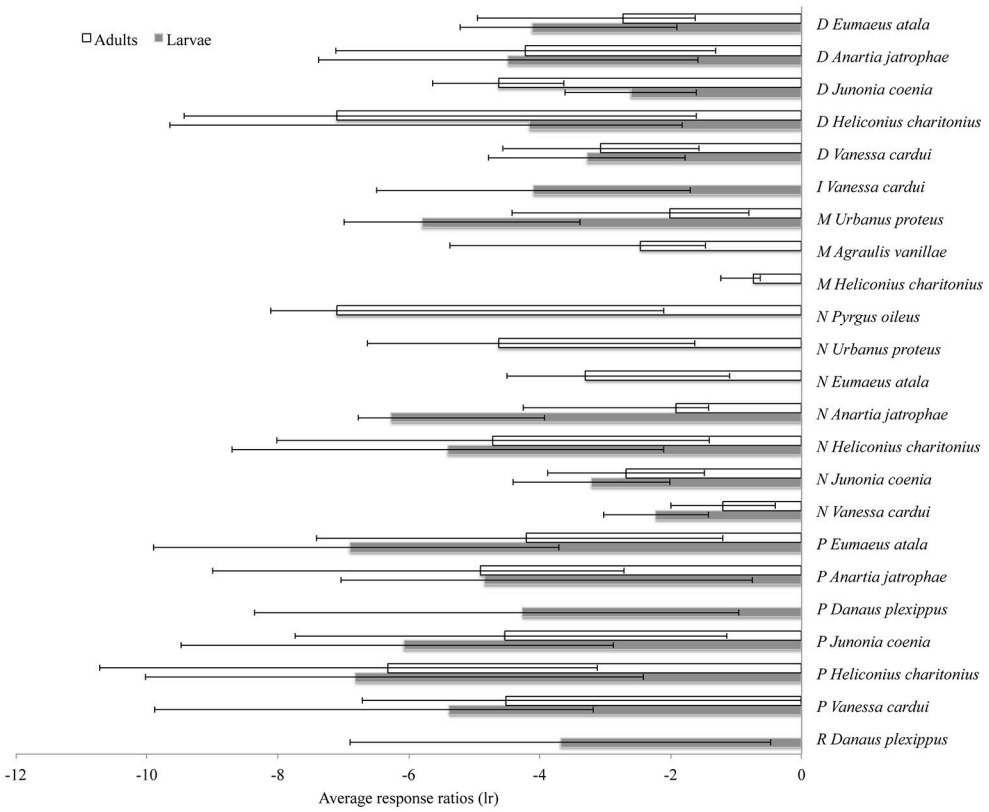


FIGURE 1 | The effect of insecticides (D, dichlorvos; I, imidacloprid; M, malathion; N, naled; P, permethrin; R, resmethrin) on larvae and adults of diurnal Lepidoptera, specimens are grouped in alphabetic order by family and then by species. Data are presented as average response ratios (*lr*) of treated-to-control group. The extremities of the bars indicates the minimum and maximum effects.

on this topic, including the knowledge of the sub-lethal effects of PPPs on NTOs, in accordance with the opinion expressed in 2015 by EFSA's researchers (EFSA, 2015) who made recommendations for further toxicity studies on PPPs, using Lepidoptera larvae as representatives of herbivorous species of non-target arthropods.

AUTHOR CONTRIBUTIONS

Conception and design of study: BM. Acquisition of data: RM, GS, and LR. Analysis and/or interpretation of data and drafting the manuscript: BM and RM. Revising the manuscript critically for important intellectual content and editing assistance: BM, LR, and RM.

ACKNOWLEDGMENTS

This work was partially supported by the 2012-ATE-0322 project, financed by the University of Palermo and BM self-funding researches. The authors wish to acknowledge the reviewers for their detailed and helpful comments to the manuscript. We thank Dr. Burket for English corrections that help improving the manuscript as well as his constructive comments.

REFERENCES

AAVV (2015). *Global Crop Protection Chemicals (Pesticides) Market – Growth, Trends and Forecasts* (2016–2021). Hyderabad: Mordor Intelligence.

Aktar, M. D. W., and Sengupta, D., Chowdhury, A. (2009). Review article–Impact of pesticides use in agriculture: their benefits and hazards. *Interdisc.Toxicol.* 2, 1–12. doi: 10.2478/v10102-009-0001-7

Bargar, T. A. (2012). Risk assessment for adult butterflies exposed to the mosquito control pesticide naled. *Environ. Toxicol. Chem.* 31, 885–891. doi: 10.1002/etc.1757

Bonebrake, T. C., Ponisio, L. C., Boggs, C. L., and Ehrlich, P. R. (2010). More than just indicators: a review of tropical butterfly ecology and conservation. *Biol. Conserv.* 143, 1831–1841. doi: 10.1016/j.biocon.2010.04.044

Boriani, L., Burgio, G., Marini, M., and Genghini, M. (2005). Faunistic study on butterflies collected in Northern Italy rural landscape. *Bull. Insectol.* 58, 49–56.

Collins, N. M., and Morris, M. G. (1985). *Threatened Swallowtail Butterflies of the World The IUCN Red Data Book*. Cambridge: IUCN.

EEA (2013). *The European Grassland Butterfly Indicator: 1990–2011*. EEA Technical Report 11/2013. Publications Office of the European Union.

EFSA (2010). Guidance of EFSA. Application of systematic review methodology to food and feed safety assessments to support decision making. *EFSA J.* 8:1637. doi: 10.2903/j.efsa.2010.1637

EFSA (2015). PPR Panel (EFSA Panel on Plant Protection Products and their Residues). Scientific Opinion addressing the state of the science on risk assessment of plant protection products for non-target arthropods. *EFSA J.* 13:3996. doi: 10.2903/j.efsa.2015.3996

Fiedler, K. (1996). Host-plant relationships of lycaenid butterflies: large-scale patterns, interactions with plant chemistry, and mutualism with ants. *Entomol. Exper. Appl.* 80, 259–267. doi: 10.1111/j.1570-7458.1996.tb00931.x

Fiedler, K. (1998). Diet breadth and host plant diversity of tropical - vs. temperate - zone herbivores: South-East Asian and West Palaearctic butterflies as a case study. *Ecol. Entomol.* 23, 285–297. doi: 10.1046/j.1365-2311.1998.00132.x

Fileccia, V., Santorsola, S., Arpaia, S., and Manachini, B. (2015). Seasonal patterns in butterfly abundance and species diversity in five characteristic habitats in sites of community importance in sicily (Italy). *Bull. Insectol.* 68, 91–102.

Goulson, D. (2013). Review: an overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol.* 50, 977–987. doi: 10.1111/1365-2664.12111

Griffis, K. L., Mann, S. S., and Wagner, M. R. (2001). "The suitability of butterflies as indicators of ecosystem condition: a comparison of butterfly diversity across stand treatments in northern Arizona," in *5th Biennial Conference of Research on the Colorado Plateau, Conference Proceedings,* eds C. van Riper, K. A. Thomas, and M. A. Stuart (Report Series USGSFRESC/COPL/2001/24), 125–135.

Gullan, P. J., and Cranston, P. S. (2008). *Lineamenti di Entomologia*. Bologna: Zanichelli.

Hahn, M., Schotthöfer, A., Schmitz, J., Franke, L. A., and Brühl, C. A. (2015). The effects of agrochemicals on Lepidoptera, with a focus on moths and their pollination service in field margin habitats. *Agric. Ecosys. Environ.* 207, 153–162. doi: 10.1016/j.agee.2015.04.002

Hoang, T. C., Pryor, R. L., Rand, G. M., and Frakes, R. A. (2011). Use of butterflies as nontarget insect test species and the acute toxicity and hazard of mosquito control insecticides. *Environ. Toxicol. Chem.* 30, 997–1005. doi: 10.1002/etc.462

Krischik, V., Rogers, M., Gupta, G., and Varshney, A. (2015). Soil-applied imidacloprid translocates to ornamental flowers and reduces survival of adult *Coleomegillamaculata*, *Harmoniaaxyridis*, and *Hippodamia convergens* lady beetles, and larval *Danaus plexippus* and *Vanessa cardui* butterflies. *PLoS ONE* 10:e0119133. doi: 10.1371/journal.pone.0119133

Nicholls, C. I., and Altieri, M. A. (2013). Plant biodiversity enhances bees and other insect pollinators in agroecosystems. A review. *Agron. Sustain. Dev.* 33, 257–274. doi: 10.1007/s13593-012-0092-y

Oberhauser, K. S., Brinda, S. J., Weaver, S., Moon, R. D., Manweiler, S. A., and Read, N. (2006). Growth and survival of monarch butterflies (Lepidoptera: Danaidae) after exposure to permethrin barrier treatments. *Environ. Entomol.* 35, 1626–1634. doi: 10.1093/ee/35.6.1626

Oberhauser, K. S., Manweiler, S. A., Lelich, R., Blank, M., Batalden, R. V., and De Anda, A. (2009). Impacts of ultra-low volume resmethrin applications on non-target insects. *J. Am. Mosq. Control Assoc.* 25, 83–93. doi: 10.2987/08-5788.1

Pierce, N. E., Braby, M. F., Heath, A., Lohman, D. J., Mathew, J., Rand, D. B., et al. (2002). The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). *Annu. Rev. Entomol.* 47, 733–771. doi: 10.1146/annurev.ento.47.091201.145257

Popp, J., Pető, K., and Nagy, J. (2012). Pesticide productivity and food security. A review. *Agron. Sustain. Dev.* 33, 243–255. doi: 10.1007/s13593-012-0105-x

Salvato, M. H. (2001). Influence of mosquito control chemicals on butterflies (Nymphalidae, Lycaenidae, Hesperiidae) of the Lower Florida Keys. *J. Lepidopt. Soc.* 55, 8–14.

Sciarra, D., Foderà, I., and Ceriani, N. (2015). *Stop Pesticidi: Analisi dei Residui di Pesticidi Negli Alimenti e Buone Pratiche Agricole–Dossier Legambiente*. Ufficio Stampa; Legambiente.

Wang, K., Hao, J., and Zhao, H. (2013). Characterization of complete mitochondrial genome of the skipper butterfly, *Celaenorrhinus maculosus* (*Lepidoptera*: *Hesperiidae*). *Mitochondrial DNA* 26, 690–691. doi: 10.3109/19401736.2013.840610

Zakharov, E. V., Caterino, M. S., and Sperling, F. A. H. (2004). Molecular phylogeny, historical biogeography and divergence time estimatesfor swallowtail butterflies of the genus *Papilio* (*Lepidoptera*: *Papilionidae*). *Syst. Biol.* 53, 193–215. doi: 10.1080/10635150490423403

Zhong, H., Hribar, L. J., Daniels, J. C., Feken, M. A., Brock, C., and Trager, M. D. (2010). Aerial ultra-low-volume application of naled: impact on non target imperiled butterfly larvae (*Cyclargus thomasi bethunebakeri*) and efficacy against adult mosquitoes (*Aedes taeniorhynchus*). *Environ. Entomol.* 39, 1961–1972. doi: 10.1603/EN10089

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Mulé, Sabella, Robba and Manachini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

REVIEW
published: 02 November 2016
doi: 10.3389/fenvs.2016.00071

Contamination of the Aquatic Environment with Neonicotinoids and its Implication for Ecosystems

Francisco Sánchez-Bayo[1]*, *Koichi Goka*[2] *and Daisuke Hayasaka*[3]

[1] Faculty of Agriculture and Environment, The University of Sydney, Eveleigh, NSW, Australia, [2] National Institute for Environmental Studies, Tsukuba, Japan, [3] Faculty of Agriculture, Kindai University, Nara, Japan

The widespread use of systemic neonicotinoid insecticides in agriculture results first in contamination of the soil of the treated crops, and secondly in the transfer of residues to the aquatic environment. The high toxicity of these insecticides to aquatic insects and other arthropods has been recognized, but there is little awareness of the impacts these chemicals have on aquatic environments and the ecosystem at large. Recent monitoring studies in several countries, however, have revealed a world-wide contamination of creeks, rivers and lakes with these insecticides, with residue levels in the low μg/L (ppb) range. The current extent of aquatic contamination by neonicotinoids is reviewed first, and the findings contrasted with the known acute and chronic toxicity of neonicotinoids to various aquatic organisms. Impacts on populations and aquatic communities, mostly using mesocosms, are reviewed next to identify the communities most at risk from those that undergo little or no impact. Finally, the ecological links between aquatic and terrestrial organisms are considered. The consequences for terrestrial vertebrate species that depend mainly on this food source are discussed together with impacts on ecosystem function. Gaps in knowledge stem from difficulties in obtaining long-term experimental data that relates the effects on individual organisms to impacts on populations and ecosystems. The paper concludes with a summary of findings and the implications they have for the larger ecosystem.

Keywords: macroinvertebrates, ecological impacts, systemic insecticides, imidacloprid, meta-analysis, review

OPEN ACCESS

Edited by:
Carsten A. Brühl, University of Koblenz and Landau, Germany

Reviewed by:
Alexa C. Alexander, Environment Canada, Canada
Yong Liu, Hunan Plant Protection Institute, China

***Correspondence:**
Francisco Sánchez-Bayo
sanchezbayo@mac.com;
francisco.sanchez-bayo@sydney.edu.au

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *15 August 2016*
Accepted: *17 October 2016*
Published: *02 November 2016*

Citation:
Sánchez-Bayo F, Goka K and Hayasaka D (2016) Contamination of the Aquatic Environment with Neonicotinoids and its Implication for Ecosystems. Front. Environ. Sci. 4:71. doi: 10.3389/fenvs.2016.00071

INTRODUCTION

Neonicotinoids are a novel class of chemical insecticides derived from the natural toxin nicotine. The first compound that was launched to the market in the early 1990s, imidacloprid, was hailed as the solution to the environmental problems caused by older insecticides such as organochlorines and organophosphates, e.g., spray drift onto non-target areas (Siebers et al., 2003), broad-spectrum toxicity to most organisms (Brown, 1978), fish kills (Fox and Matthiessen, 1982), bioaccumulation in fatty tissues (Matthiessen et al., 1982) and poisoning effects on the applicators (Cataño et al., 2008), among others.

Because they are systemic, neonicotinoids are typically applied to the roots of the crop plant, avoiding thus the need for spraying and contaminating nearby land by drift, although they can also be applied as foliar sprays (Elbert et al., 2008). In addition, the selectivity of neonicotinoids toward arthropods, and insects in particular, was an achievement only paralleled by the pyrethroids (derived from the natural toxin pyrethrum). However, unlike the latter insecticides,

neonicotinoids are not toxic to either fish or zooplankton species, a great advantage for using them in environmental programs. Thirdly, their hydrophilic properties avoided any chance of bioaccumulation in organisms, and furthermore they were harmless to mammals (Tomizawa and Casida, 2005). These features provided safety to both environment and operators, mainly farmers, and became the key selling points in marketing (Jeschke and Nauen, 2008). Within a decade, imidacloprid was the top selling insecticide in the world, having displaced older chemistries (Jeschke et al., 2011). Newly developed neonicotinoids followed suit, to the point that they now constitute the largest group of insecticides in the global market (Simon-Delso et al., 2015).

Their marketing success, however, was tarnished by their association with honey bee failures in France (van der Sluijs et al., 2013). Many people now have become aware of the existence of neonicotinoids through reports in the media and the internet about bee declines. Hundreds of research papers have been written on this topic in recent years (Osborne, 2012), and the evidence suggests that neonicotinoids impacts on bees and other pollinators cannot be ignored (EFSA, 2013). Consequently, authorities have started to impose measures in Europe to reduce their use in crops that attract bees, like rapeseed (canola), sunflower, and maize (European Commission, 2013), and some countries (i.e., France and Germany) have recently banned the use of seeds treated with neonicotinoids (Garric and Hir, 2016).

As the debate about neonicotinoids has been focused on bees not many people are aware of their impacts on aquatic ecosystems. Yet they pose threats to this environment (Sánchez-Bayo, 2014), more subtle perhaps but broader in scope when we analyse the consequences for the larger aquatic ecosystem.

This paper is a review of current knowledge about the toxicity of neonicotinoids to aquatic species, starting with their effects at the individual level, discussing their impacts on populations and aquatic communities, and concluding with the consequences that these impacts have on ecosystems. In addition, a meta-analysis of the contamination of freshwater systems with neonicotinoids to date adds a global perspective to the issues. There is urgency in assessing the advantages and disadvantages of the widespread use of this class of insecticides, so as not to repeat the mistakes of the past (Krebs et al., 1999).

EFFECTS AT THE ORGANISMAL LEVEL

The first neonicotinoid launched to the market, imidacloprid, had very low acute toxicity to the standard aquatic species used in regulatory testing of chemicals. The 48-h median lethal concentration (LC50) for the waterflea *Daphnia magna* was between 10 and 85 mg/L (ppm), which is several orders of magnitude higher than the LC50 of pyrethroids, organophosphorus and carbamate insecticides to the same species (Song et al., 1997; Tomlin, 2009). Similarly, the 96-h LC50 for fish species were in the range 83–281 mg/L for rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Ding et al., 2004; Tomlin, 2009). These data suggested that neonicotinoids would not have major impacts, if any, on aquatic ecosystems.

It was later found that other aquatic taxa were much more sensitive to imidacloprid than the standard test species. Thus, freshwater ostracods have 48-h LC50s in the range 185–719 μg/L (ppb), which are 50–120 times lower than that of *D. magna* (Sánchez-Bayo and Goka, 2006b). Moreover, the LC50s for the amphipod *Hyalella azteca* are between 115 and 7 μg/L (ppb) depending on whether the exposure is for 2 or 28 days, and for midge larvae (*Chironomus tentants*) LC50s are in the range from 5 to 0.9 μg/L for the respective 4 and 28-day exposures (Stoughton et al., 2008). The latter values are four orders of magnitude lower than those for *D. magna*, indicating that somehow the initial toxicity assessment of this insecticide was flawed. Survival of midge larvae (*Chironomus riparius*) is also reduced in waters contaminated with mixtures of neonicotinoids (imidacloprid, thiacloprid) and pyrethroid insecticides (deltamethrin and esfenvalerate) (Kunce et al., 2015).

One particular aspect of neonicotinoids became apparent only after years of testing: median toxicity values varied significantly depending on the time of exposure. As mentioned above, the estimated LC50s for amphipods and midge larvae were one or two orders of magnitude lower for exposures of 28 days compared to standard exposures of 2 or 4 days. This translates in a large acute/chronic toxicity ratio, which for the mayfly *Cloeon dipterum* is 800 times when exposed for 28 days to imidacloprid (van den Brink et al., 2016). For the freshwater ostracod *Cypridopsis vidua*, the difference in LC50 between 2- and 5-day exposures is three orders of magnitude! (Sánchez-Bayo, 2009). This trend toward lower LC50s with increasing exposure time has been confirmed for several other species, including *D. magna, Gammarus* amphipods, black fly larvae, alderflies, mayfly and dragonfly nymphs (Beketov and Liess, 2008a; Roessink et al., 2013) when exposed to imidacloprid, thiamethoxam or thiacloprid (**Figure 1**). The consequence is an apparent "delayed mortality" (Beketov and Liess, 2008a), which can be observed in mesocosm trials that use a single pulse exposure: most of the organisms do not die immediately but start dying in large numbers after a week, and their populations disappear completely after a few weeks (Sánchez-Bayo and Goka, 2006a; Hayasaka et al., 2012a).

The physiological mechanism responsible for such unusual toxicological response is based on the agonistic mode of action of this class of chemicals, and was deduced by Tennekes (2010a) based on the model of Druckrey and Küpfmüller (1949). Neonicotinoids bind irreversibly to the nicotinic acetylcholine receptors (nAChR) embedded in the synaptic membranes of neurons, and their activation elicits a continuous electric impulse that eventually leads to the death of the neuron. The neuronal death toll accumulates as more and more chemical molecules bind to other nAChRs until the organism cannot cope with the damage and dies (Rondeau et al., 2014). Although an antagonistic mode of action on the same receptors has been reported for thiacloprid and thiamethoxam in *Lymnaea stagnalis* snails (Vehovszky et al., 2015), this inhibition of neurotransmission results in a similar outcome.

Aquatic organisms are constantly being exposed to residues of chemicals present in water, a medium from which they

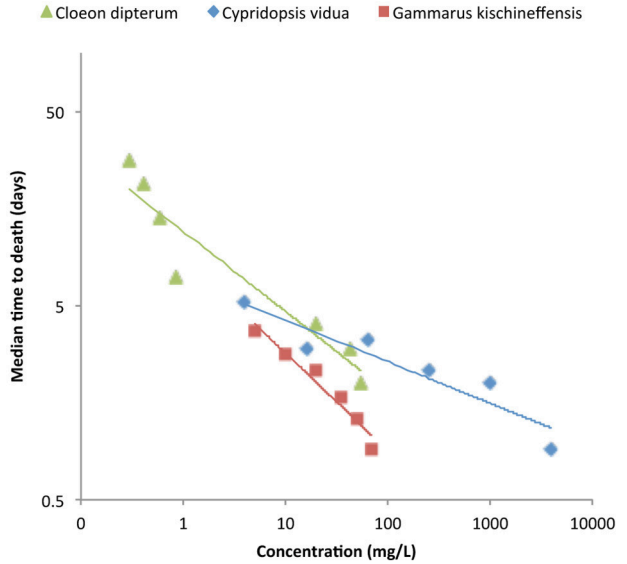


FIGURE 1 | Time-cumulative mortality of three aquatic species exposed to neonicotinoids: mayfly *Cloeon dipterum* to thiacloprid (green triangles), ostracod *Cypridopsis vidua* to imidacloprid (blue diamonds) and amphipod *Gammarus kischineffensis* to thiamethoxam (red squares). Data sources: mayfly (van den Brink et al., 2016); ostracod (Sánchez-Bayo, 2009); amphipod (Uğurlu et al., 2015).

cannot escape. The time to reach the organism's death threshold depends on the internal concentration of insecticide, which in turn depends on its external concentration and the kinetics and detoxification ability of each species (Escher et al., 2011). The latter explains the enormous differences in susceptibility to this class of insecticides by various aquatic and terrestrial taxa, which range several orders of magnitude among insects and crustaceans alone (Morrissey et al., 2015).

The main difference between this mode of action and that of other pesticides is that effects are cumulative with time, because neurons do not regenerate. It has been termed time-cumulative toxicity (Tennekes and Sánchez-Bayo, 2013) to distinguish it from the more common toxicological response of insecticide inhibitors (e.g., organophosphorus, pyrethroids), which may bind irreversibly to specific receptors or enzymes but whose effects are temporary and can be reversed once the target receptors or enzymes are regenerated (Matsumura, 1985).

Aside from mortality, exposure to neonicotinoids causes a number of sublethal effects on aquatic organisms, such as feeding inhibition (Alexander et al., 2007; Kreutzweiser et al., 2007; Nyman et al., 2013), impaired movement (Motobayashi et al., 2012), reduced fecundity (Böttger et al., 2013), reduced body size in mayflies (Alexander et al., 2008) and fish (Hayasaka et al., 2012a) and immune-suppression in fish (Sánchez-Bayo and Goka, 2005). Downstream drift also occurs probably as an avoidance response to toxic conditions (Beketov and Liess, 2008b). All these effects were ignored for years, as the focus of neonicotinoid research was on bees, not on aquatic organisms. Obviously, some of these sublethal effects can be reversed if they do not rely directly upon the nervous system.

IMPACTS AT THE POPULATION LEVEL

Measurements of acute toxicity such as LC50s are useful to determine the potency of a chemical. Equally useful are the estimations of lowest effect concentrations (LOECs) based on observations of chronic exposure, although they are less accurate and reliable. What matters is to protect the populations of as many species as possible so as to maintain the integrity of the aquatic ecosystem services. To achieve that goal, it is imperative to know the range of sensitivities amongst species in different taxonomic groups, so that an evaluation of risks can be made. For aquatic species, toxicity data are scarce for all neonicotinoids except for imidacloprid and, to a lesser extent, thiacloprid. Therefore, assessments of risks and water quality thresholds for neonicotinoids are currently based on the acute toxicity of imidacloprid, mostly derived from short-term exposures of 2 or 4 days (Morrissey et al., 2015) and some chronic data (Smit et al., 2015).

The ECOTOX database of the US Environmental Protection Agency (EPA) provides LC50s of imidacloprid for 57 aquatic species belonging to several taxonomic groups (9 classes in 4 phyla). Sensitivities span six orders of magnitude, from the most susceptible mayflies (LC50 ~ 1 ppb) to the most tolerant fish (LC50 ~ 650 ppm) (**Figure 2**). It is apparent that the most susceptible species are aquatic insects, followed by crustaceans such as amphipods, ostracods and shrimps, then tubicifid worms and mussels. All cladoceran crustaceans (waterfleas) are very tolerant except perhaps *Ceriodaphnia dubia*, which is as sensitive as ostracods. Waterfleas are, therefore, not representative of other invertebrate taxa for imidacloprid nor any other neonicotinoid compound (Beketov and Liess, 2008a; Daam et al., 2013; Hayasaka et al., 2013).

Species sensitivity distributions (SSD, **Figure 2**) have been used by government agencies in some countries to derive water quality thresholds that protect their aquatic environment. In the Netherlands and other European countries the protective level for short-term peak concentrations of imidacloprid is 0.2 µg/L, whereas for long-term exposures the threshold is 8.3 ng/L (Smit et al., 2015). In the United States, the chronic invertebrate Aquatic Life Benchmark is 1.05 µg/L, in Canada 0.23 µg/L (CCME, 2007; Anderson et al., 2015) and in Sweden 13 ng/L (Kreuger et al., 2010). Thresholds for other neonicotinoids are about the same order of magnitude in the US, but most countries have not established yet any regulation concerning neonicotinoids, while many still base their ecological assessments on the misleading toxicity data for *Daphnia* and fish.

The above regulatory thresholds are only a guide. Unlike with previous pesticides, protective levels for neonicotinoids cannot be achieved by setting a concentration benchmark because, as already explained, the effects of neonicotinoids increase with exposure time. An alternative is to assess the impact on populations using the predicted affected fraction (PAF) of species, which is determined by comparing waterborne residue levels from monitoring surveys with the SSD. Data on water residues for these compounds have been gathered in the past decade; prior to 2005 only a few surveys found some imidacloprid in a watershed and two streams of New York State (USGS, 2002;

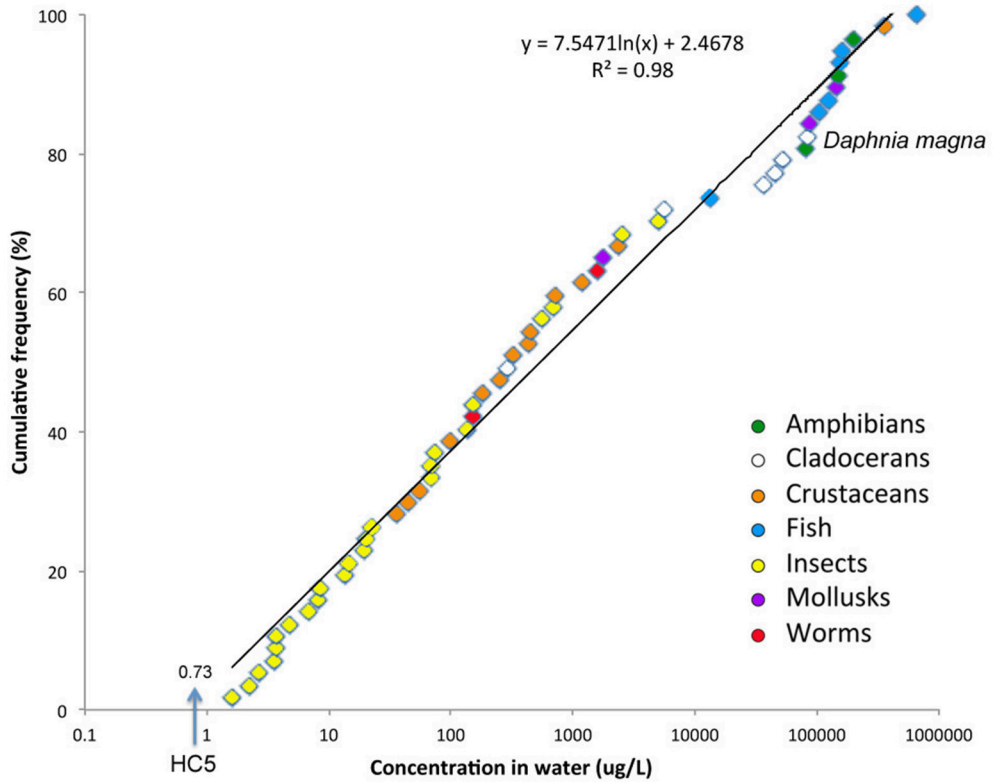


FIGURE 2 | Species sensitivity distribution (SSD) for acute toxicity of imidacloprid (LC50) to aquatic organisms. The hazard concentration affecting 5% of species (HC5, μg/L) is indicated. Source: ECOTOX database https://cfpub.epa.gov/ecotox/.

Phillips and Bode, 2004) and in drains from potato fields in Canada (Denning et al., 2004; CCME, 2007). A meta-analysis of all residue data from 11 countries available to date (Table S1) revealed the following:

(i) up to six neonicotinoids are currently present in water bodies all over the world. Average concentrations were similar for all compounds, ranging from 0.08 μg/L (dinotefuran) to 0.73 μg/L (imidacloprid); the highest concentrations detected so far were for imidacloprid and thiamethoxam (320 and 225 μg/L, respectively) (**Figure 3**).

(ii) average residue levels have increased over the past 15 years, with highest rates of increase for clothianidin and thiamethoxam reflecting the worldwide trend in usage of these two compounds (Simon-Delso et al., 2015; **Figure 4A**).

(iii) the frequency of detection varies widely from country to country, with 100% detections for some compounds in several regions. On average neonicotinoid detections were found in 13% (acetamiprid) to 57% (dinotefuran) of all waters, and they also showed an increasing trend with time; again, the highest increases were for clothianidin and thiamethoxam (**Figure 4B**).

Contrasting these data with the protective levels established in some countries, the average concentration of all neonicotinoids in water exceeded the European guidelines 27% of the time, and the Canadian and United States guidelines 66 and 79% of the time, respectively, whereas maximum concentrations can exceed the European guidelines 35% of the time (Morrissey et al., 2015).

These findings are of concern. The increasing trend in detections is obviously due to two factors: (i) a major effort in looking for these compounds in recent times, which contrasts with the absence of data in previous years; and (ii) better analytical capabilities, with current limits of detection around 1 ng/L or less using either HPLC (Sánchez-Bayo and Hyne, 2014) or LC-MS/MS instrumentation (Hladik and Calhoun, 2012; Yamamoto et al., 2012). However, the increasing residue levels are of great concern, as they indicate that residues in soil, where most of these insecticides are applied, are accumulating over the years. Indeed, there is evidence that such accumulation is happening in countries with a long history of using seeds treated with imidacloprid (Jones et al., 2014; Douglas and Tooker, 2015), although residues of thiamethoxam or clothianidin may plateau if crop rotation is used over a few years (Schaafsma et al., 2016). This accumulation results primarily from the fact that 80–90% of the insecticide in the coated-seeds and granules remains in the soil at the end of the cropping season (Sur and Stork, 2003; Goulson, 2013), and dissipation from soil is slower than in water: half-lives of neonicotinoids in soil are between 50 and 600 days for the four most commonly used compounds (Bonmatin et al., 2015), whereas photolytic hydrolysis in water can dissipate waterborne residues in a few

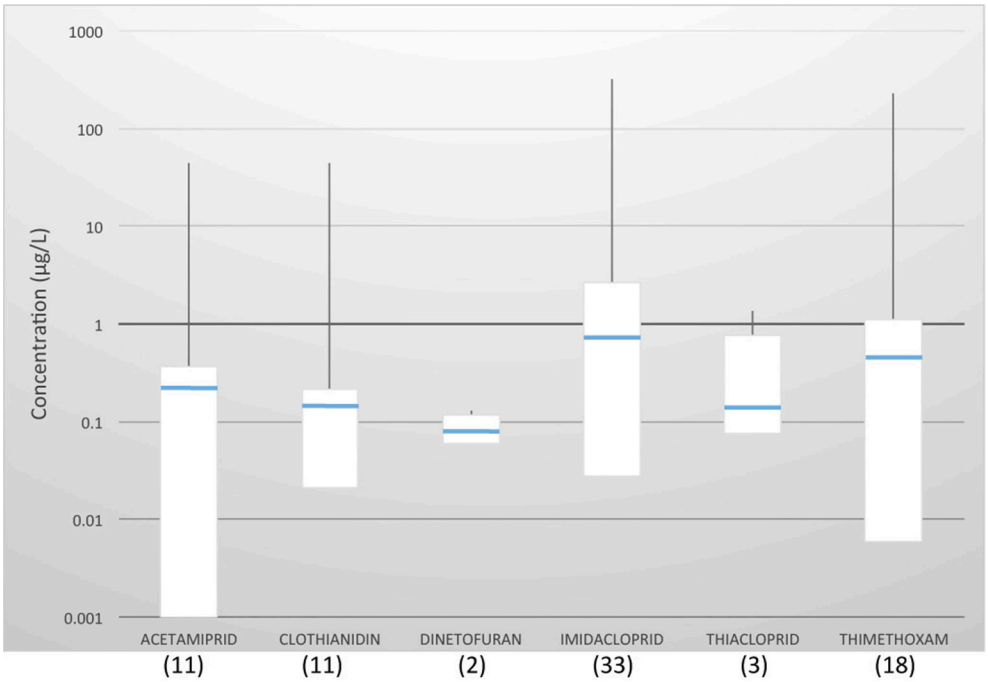


FIGURE 3 | Worldwide survey of neonicotinoid residues in water. The number of surveys reporting each chemical is in brackets. Boxes contain the residues between the first and third quartile; blue lines indicate the geometric mean; vertical lines show the outliers. Sources: see Table S1.

days (Phong et al., 2009; Thuyet et al., 2011). Degradation in sediments is faster for newly developed compounds like cycloxaprid (Liu et al., 2015). Up to 6% of imidacloprid residues in soil can be transported in runoff after storm events (Thuyet et al., 2012), but most of the residual chemical would remain in the applied field, from where it moves readily into ground waters, particularly thiamethoxam, imidacloprid, clothianidin (González-Pradas et al., 2002; Miranda et al., 2011; Bajeer et al., 2012) and dinotefuran (Kurwadkar et al., 2014). The increasing use of products containing neonicotinoids and their repeated application as coated seeds in agricultural fields (Douglas and Tooker, 2015) adds every year a new layer of residues to the soil, and hence to the waters, where residue levels are a reflection of those present in soil at any time (Hladik et al., 2014; Schaafsma et al., 2015).

By comparing the distribution of waterborne residues of imidacloprid to its SSD, estimations of the PAF are made to assess its current impact on aquatic organisms, i.e., the loss of half the populations exposed (**Figure 5**). Surveys to date indicate that up to 40% of species are being seriously affected in streams of Maryland, where average residue levels are 5.4 µg/L and can reach 131 µg/L (Johnson and Pettis, 2014), and a similar proportion in draining ditches from greenhouses in Sweden (average 3.2 µg/L and highest 89 µg/L) (Kreuger et al., 2010). In three agricultural valleys of California, imidacloprid is currently affecting up to 11% of aquatic species (Starner and Goh, 2012), and in the Sydney basin up to 14% of species are being affected in streams that receive water from turf farms (Sánchez-Bayo and Hyne, 2014). Only streams and estuaries contaminated mostly with urban runoff, e.g., San Francisco (Weston et al., 2015), have minimal number of species affected. One can expect similar impacts for the other neonicotinoid residues, although it is not possible to assess them at this stage so long as the data available are insufficient.

This preliminary assessment is only based on the acute toxicity data (**Figure 2**) as determined in laboratories. For more realistic assessments of the long-term impacts, field and mesocosm studies are required, as explained in the next section.

IMPACTS ON AQUATIC COMMUNITIES

Some 22 studies on the impacts of neonicotinoids on aquatic communities have been conducted to date. Most of them comprise mesocosms that used imidacloprid, with five studies using thiacloprid and one acetamiprid in addition to those two compounds (Table S2). These studies were carried out in Japan (rice mesocoms), Portugal (field trials), Canada, and Germany (streams and microcosms). The most striking feature of these studies is their consistency in reporting population and community effects at levels well below the LC50s of the aquatic species tested. This is unusual, since field or mesocosms studies under realistic scenarios typically report fewer impacts of pesticides and other toxicants than in closed laboratory conditions (Cleveland et al., 2002). Reduced exposures, due mainly to chemical losses by microbial degradation, hydrolysis and other environmental factors, are usually responsible for the lesser impacts under field conditions (Maund et al., 1997).

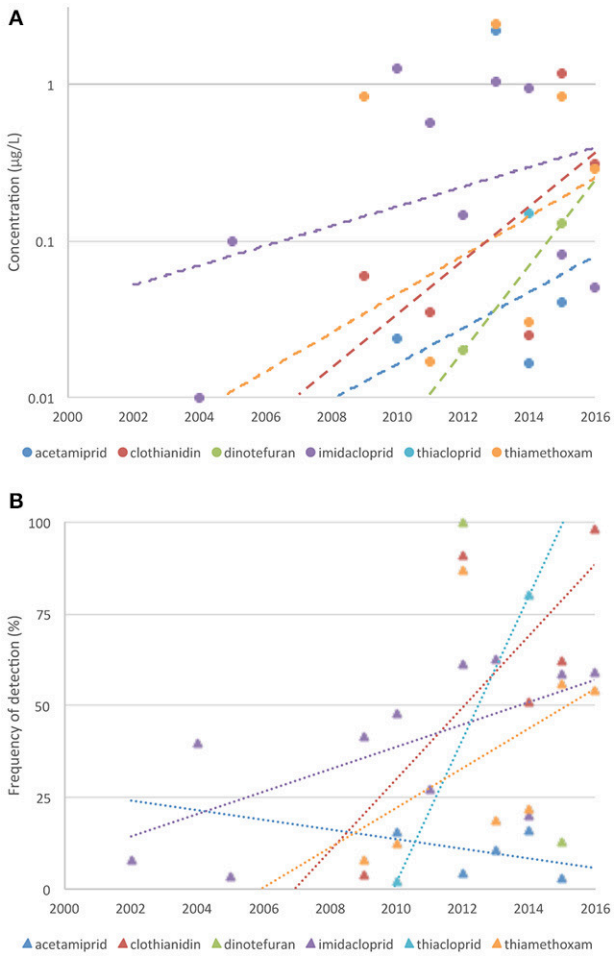


FIGURE 4 | Temporal trends of (A) concentrations of neonicotinoids in waters of the world, and (B) their frequency of detection. Sources: see Table S1.

A reduced abundance in aquatic insects is apparent when concentrations of imidacloprid in water are above 1 or 2 µg/L (Sánchez-Bayo and Goka, 2006a; Pestana et al., 2009; Hayasaka et al., 2012a; Colombo et al., 2013). Population reductions in the short-term are caused by direct toxicity, but in mesocosms such reductions affect the structure of the macroinvertebrate communities when residues are one or two orders of magnitude lower than the LC50s for most species, as more tolerant taxa tend to increase in numbers to fill the niche vacuum thus created in the ecosystem. Some of these changes are predictable. For instance, waterfleas increased in numbers when grazers such as ostracods were eliminated in rice mesocosms treated with imidacloprid (Sánchez-Bayo et al., 2007), whereas the disappearance of chironomid larvae brought about increases in *Radix* sp. snails (Colombo et al., 2013). In other cases, opportunistic predators (e.g., Heteroptera, Coleoptera, Odonata) and scavengers (e.g., Amphipoda) that feed on the moribund insects or their corpses have a temporary increase in food availability (Sánchez-Bayo and Goka, 2006a). Interestingly, the negative impacts on predatory copepod populations in rice fields are followed by upsurges of mosquitoes but not of chironomids. Consequently, the overall biodiversity of the aquatic communities is negatively affected (Pestana et al., 2009). Similar impacts are observed in mesocosms treated with thiacloprid at 3.2 µg/L or above (Kattwinkel et al., 2016). This is not surprising, as the HC5 for thiacloprid derived from outdoor stream mesocosms is 0.72 µg/L (Beketov et al., 2008), the same as that calculated for imidacloprid (HC5 = 0.73 µg/L, **Figure 2**).

Treatments of rice mesocosms with imidacloprid at different rates over 4 years in Japan resulted in community impacts that were related to the initial concentrations of this insecticide in water, from 240 to 40 µg/L. Average reductions of 46–62% were recorded among the plankton, neuston, nekton or benthos communities (**Figure 6A**), but the specific taxa groups and species affected differed from 1 year to another (Sánchez-Bayo et al., 2007; Hayasaka et al., 2012b). The greatest impacts (>45% reductions) occurred in ostracods, mayflies and snails, followed by chironomids, dragonflies, damselflies and some Hemiptera predators such as Corixidae, Mesoveliidae and *Anthocoris* sp. Emergence of dragonflies is also reduced by more than 80% (Jinguji et al., 2013). By contrast, waterfleas increased by 75% and Diptera larvae (excluding chironomids) by 15%. A similar trial in a rice field in Portugal measured initial concentrations of 52 µg/L imidacloprid in water, and average weighted concentrations of 8 µg/L, which were estimated to affect 40–63% of the aquatic species (Daam et al., 2013). These observations are consistent with the impacts in Canadian stream mesocosms (**Figure 6B**), where weekly imidacloprid pulses at 2 or 20 µg/L had the highest reductions on worms (75%), caddisflies (70%), mayflies and stoneflies (both 68%) (Pestana et al., 2009).

Moreover, many of these populations are decimated and their recovery is either slow or, if there is competition with other species, it does not take place (Liess et al., 2013). Recovery of most populations only occurs when the neonicotinoid concentrations in water or sediment are below 1 µg/L (Hayasaka et al., 2012b), whereas many univoltine or semivoltine species do not recover at all. Nor does the structure of the communities revert to the original situation, because some species disappear while others take over and increase in numbers (Beketov et al., 2008; Hayasaka et al., 2012a). These impacts contrast with those caused by other pesticides, which tend to produce a large initial mortality upon target and non-target populations alike but allow the recovery of the species affected within a few weeks (van den Brink et al., 1996; Brock et al., 2010). Thus, aquatic communities of rice paddies recovered completely in a week after foliar application of the insecticide etofenprox took place (Sánchez-Bayo et al., 2007). The reason for this difference lies in the delayed mortality after chronic exposure to very low concentrations of neonicotinoids in water or sediments and, for more tolerant species like *Gammarus roeseli*, in their reduced fecundity under similar conditions (Böttger et al., 2013). By contrast, many pyrethroids and organophosphates (with the exception of persistent compounds like chlorpyrifos) do not produce time-cumulative mortality (Parsons and Surgeoner, 1991) since their exposure is limited in time (Lahr et al., 2000; Medina et al., 2004).

Apart from the lethal effects on communities, blackfly larvae (*Simulium latigonium*), mayfly nymphs (*Baetis rhodani*)

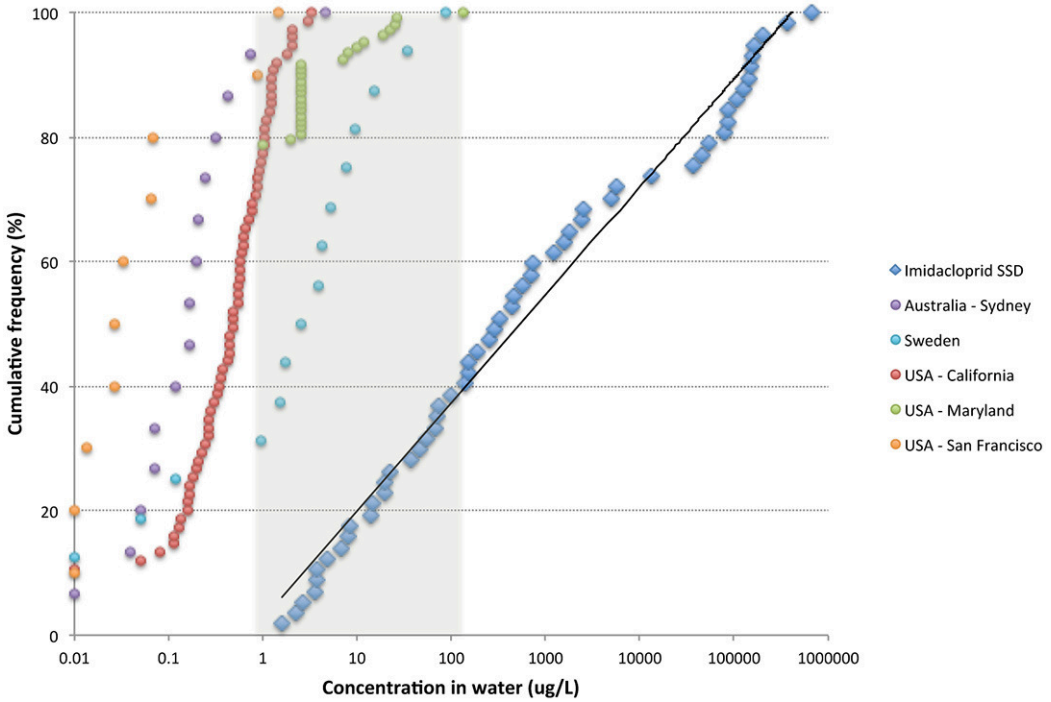


FIGURE 5 | Distribution of waterborne residues of imidacloprid in several countries contrasted with the acute SSD for this compound. Data sources: Australia (Sánchez-Bayo and Hyne, 2014); Sweden (Kreuger et al., 2010); USA California (Starner and Goh, 2012); USA Maryland (Johnson and Pettis, 2014); USA San Francisco (Weston et al., 2015).

and amphipods (*Gammarus pulex*) drift downstream when concentrations of various neonicotinoids in water are lower than 1/10 of the species LC50 (Beketov and Liess, 2008b), indicating that these organisms respond to the adverse effects of these neurotoxicants at sublethal levels, i.e., below 0.37, 0.46, and 27 µg/L of either thiacloprid, acetamiprid or imidacloprid for the respective species above.

Finally, when mayfly nymphs (*Baetis rhodani*) and *Gammarus fossarum* are exposed together to sublethal levels of thiacloprid, the amphipod increases its predation on the nymphs but reduces its shredding of litter at concentrations as low as 0.5–1 µg/L (Englert et al., 2012). Imidacloprid also reduces the litter decomposition carried out by stoneflies (*Pteronarcys dorsata*) and crane flies (*Tipula* sp.) at concentrations below 12 µg/L (Kreutzweiser et al., 2008b), probably due to a feeding inhibition effect of this insecticide, which has also been observed with other detritivores such as *Gammarus pulex* (Nyman et al., 2013) and *Eisenia fetida* earthworms (Kreutzweiser et al., 2008a). The implications of these impacts for the larger ecosystem are discussed next.

IMPACTS ON THE ECOSYSTEM

The consequences of all the above for the larger ecosystem have not been studied in detail yet. Difficulties in obtaining long-term experimental data that relates the effects on individual organisms to impacts on ecosystems prevent carrying out such studies. However, it is clear that some predictions can be made from the limited set of observations about the effects on aquatic communities reported so far. At least two main areas of concern can be identified: reduced capacity for decomposition of organic debris by aquatic organisms and starvation of insectivores and other vertebrate fauna that depend on invertebrates as a major or only food source (**Figure 7**).

Reduced Decomposition Capacity

The recycling of organic matter that falls into water bodies is an essential ecosystem function that not only provides food for a wide range of aquatic and benthic organisms but also ensures the water quality is adequate for all other organisms that use it, including ourselves.

It is well established that mayfly (Ephemeroptera), caddisfly (Trichoptera), and stonefly (Plecoptera) nymphs are the most sensitive aquatic organisms to most pollutants, so they are considered bioindicators of water quality (Morse et al., 1993; von der Ohe et al., 2007). They are shredders of leaves and other debris found at the bottom of creeks and streams that run through forested and agricultural areas, although not the only ones: larvae of crane flies (Tipulidae), black flies (Simulidae) and other Diptera taxa perform the same function, together with amphipods, ostracods and aquatic isopods. The fact that litter decomposition by stoneflies, crane flies, mayflies and amphipods is significantly reduced by concentrations of neonicotinoids that are currently found in many aquatic environments is of

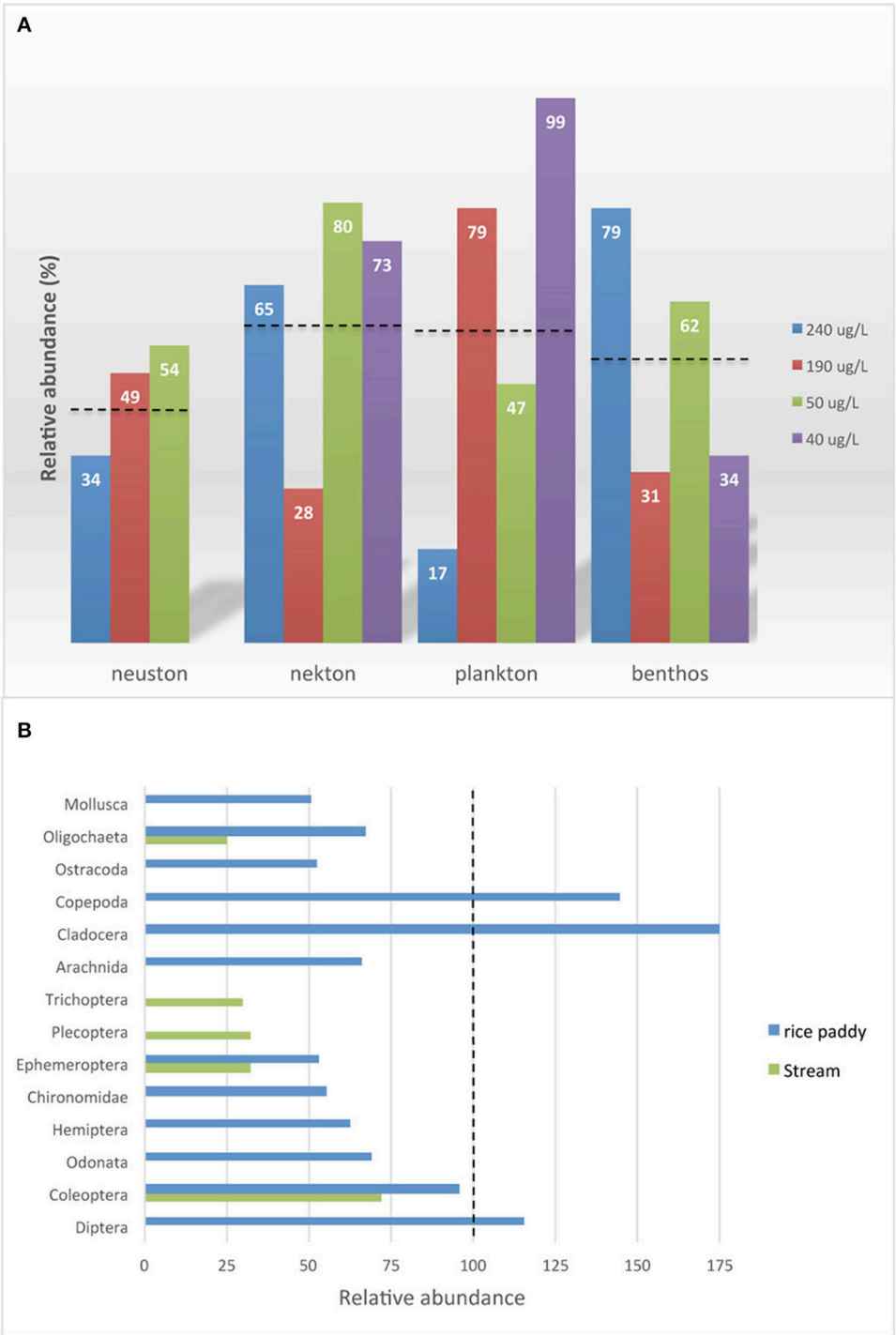


FIGURE 6 | Relative abundance with respect to controls of aquatic invertebrates in imidacloprid-treated mesocosms. (A) Communities in rice paddies for different initial concentrations of imidacloprid; dashed lines indicate average reductions. **(B)** Invertebrate taxa in rice paddies and streams; vertical dashed line indicates the control. Data sources: rice paddies (Sánchez-Bayo and Goka, 2006a; Sánchez-Bayo et al., 2007; Hayasaka et al., 2012a,b); streams (Pestana et al., 2009).

concern (Kreutzweiser et al., 2007). Even if some individuals may survive in depleted populations, they still will be unable to carry out the decomposition function properly due to the feeding inhibition caused by these neurotoxicants, which will render those individuals unfit to do their job. Naturally, insufficient feeding leads to reduced ability for reproduction (Böttger et al., 2013), so the long term prospects are poor for the detritivore populations affected.

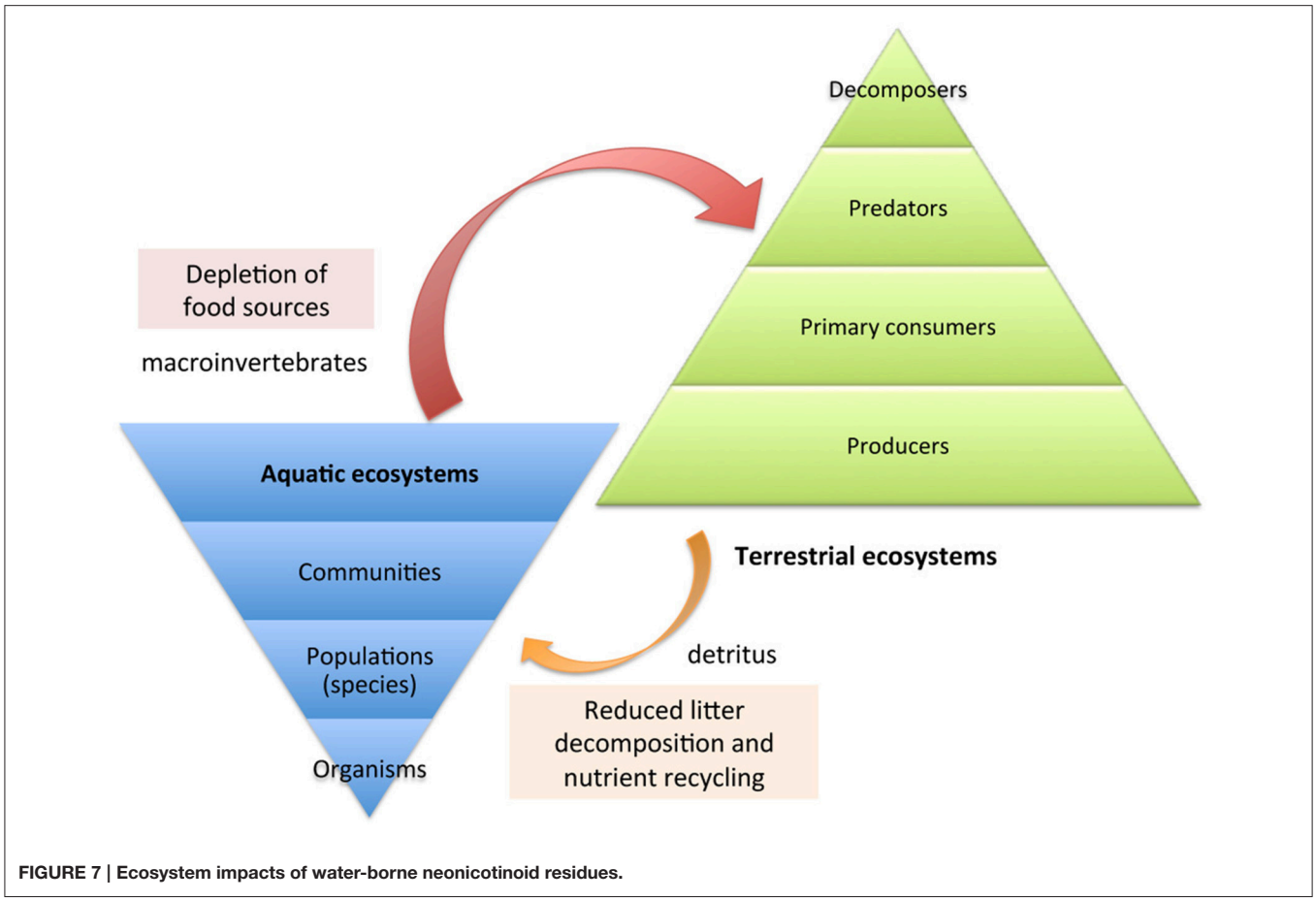


FIGURE 7 | Ecosystem impacts of water-borne neonicotinoid residues.

To many regulators of chemicals, whether mayflies or other macroinvertebrates are depleted is not important, or at least not as much as the increase in productivity that farmers may obtain from using products like neonicotinoids, although the latter benefits are questionable—see (Seagraves and Lundgren, 2012; Macfadyen et al., 2014). Just because macroinvertebrates are not seen, since they are small and live at the bottom of ponds and streams, this does not mean they can be dispensed with. As Suter and Cormier have argued, these small creatures are present in ecosystems for an important reason (Suter and Cormier, 2015).

Given that more than half of the waters are contaminated (**Figure 4**) with neonicotinoid levels that impair this important ecosystem function, higher organic and inorganic pollution can be expected wherever these insecticides are present. Microbial degradation of the debris may still occur, but it would be slower and produce undesirable by-products such as methane and sulfides (Sorrell and Boon, 1992; Kwok et al., 2005). The combined impacts by neonicotinoids and other pollutants could gradually poison the surface waters in many parts of the world.

Starvation of Insectivores and Invertebrate Feeders

Indirect impacts are a common feature of many pesticides, one that has no relation to the toxicity on the species ultimately affected (Sánchez-Bayo, 2011). Thus, neonicotinoids do not cause fish mortality directly, but because aquatic invertebrates are a rich food source for many species of fish, depletion and disappearance of this source in waters contaminated with neonicotinoids could affect fish stocks in freshwater ecosystems. In the Netherlands, where residues of imidacloprid in water are the highest in the world (Table S1), correlations between such residues and the decline of arthropod taxa such as Ephemeroptera, Odonata, Diptera and some crustaceans have been found (van Dijk et al., 2013). Although not all the declines can be blamed on neonicotinoids, because other pesticide residues are also found and can have similar impacts (Beketov et al., 2013; Vijver and van den Brink, 2014), it would seem that neonicotinoids are clearly involved.

As already mentioned, populations of aquatic species exposed to neonicotinoids often do not recover. Indeed, the overall abundance of macroinvertebrates in mesocosms treated with imidacloprid or thiacloprid was lower than that in the controls after a few months, while some species disappeared completely (Sánchez-Bayo and Goka, 2006a; Beketov et al., 2008). This suggests that recovery of the extinct populations in the following year must require re-colonization from nearby areas. The elimination of predatory species results in the increase of prey species, with some of them, like mosquitoes (**Figure 6B**), being a nuisance and a health hazard. In agricultural areas treated extensively with seeds containing neonicotinoids the chances

of re-colonization are less frequent for species that are not very mobile. Aquatic insects and invertebrate species are being removed from many land and water areas and heading toward extinction. This dire prediction is not far from the reality in some places. An entomological survey carried out in a region of Germany comprising agricultural land and a nature reserve reported a decline of 75% in flying insect abundance between 1989 and 2013 (Sorg et al., 2013). Many of these flying insects have aquatic life cycles, and their disappearance is probably due to their larvae not having survived in water. This astonishing reduction in entomofauna parallels the decline of wild bee species in North America and the British Isles (Fitzpatrick et al., 2007; Cameron et al., 2011; Woodcock et al., 2016) and butterflies in California and England (Gilburn et al., 2015; Forister et al., 2016) in the same period. It must be remembered that neonicotinoids were introduced in the early 1990s.

Riverine ecosystems are notorious for the rich biodiversity they encompass (Sánchez-Bayo, 1991). Many of the vertebrates living around rivers, lakes and ponds are insectivorous species that depend almost exclusively on aquatic invertebrates as their food source: frogs, newts, skinks and lizards, a large array of birds including passerines and waders, bats and shrews. All these animals, whether terrestrial or amphibian, draw their food from flying insects, their aquatic larvae, crustaceans and worms that live in the water environment. Consequently, the depletion of this food source must necessarily affect them (Tennekes, 2010b). To date, the only study available that makes a connection between bird declines and neonicotinoids in water was carried out in the Netherlands (Hallmann et al., 2014). The authors of that study collected information on 15 species of passerine birds in that country over 20 years since 1993, and correlated their abundance with residue concentrations of imidacloprid and other pesticide residues in water during the same period. All bird species studied were either insectivorous or fed insects and larvae to their offspring during the breeding season. The only pesticide that explained the declining trends of 14 bird species was imidacloprid, whereas other factors that were taken into consideration, such as urban or agricultural area, availability of some cereal crops, fertilizer use and others, were discarded by the statistical analysis. For the 6 species that showed a significant decline with imidacloprid residues, the average bird population decline was 3.5% per year in areas with residue levels above 20 ng/L (ppt). These levels are below the HC5 for imidacloprid (0.73 µg/L, **Figure 2**) and well below the LC50s of all aquatic insects tested. However, as demonstrated in the microcosm and mesocosm studies, they are sufficient to cause sublethal effects and delayed mortality, all of which can eliminate entire populations of invertebrates, without recovery in many cases.

Starvation by depletion of food sources due to pesticides was demonstrated for gray partridges (*Perdix perdix*) in England (Potts, 1986). Also, applications of fipronil insecticide for locust control in Madagascar reduced the abundance of two species of tenrec, a skink and iguanian lizards that depended on termites as their main food source (Peveling et al., 2003). Evidence of similar impacts by neonicotinoids on vertebrate taxa other than birds does not exist because of difficulties in obtaining relevant long-term experimental data. However, if terrestrial birds, lizards and mammals can be taken as examples of what occurs in nature when pesticides reduce the food source, it is reasonable to think that other taxa that are experiencing worldwide declines, such as frogs and bats can be affected by indirect neonicotinoid impacts on the aquatic environment (Mason et al., 2013). Establishing the link between food depletion and population declines in some species is not difficult, but linking food depletion to individual chemical causes is a more challenging task.

CONCLUSION

Negative impacts of neonicotinoids in aquatic environments are a reality. Initial assessments that considered these insecticides harmless to aquatic organisms may have led to a relaxation of monitoring efforts, resulting in the worldwide contamination of many aquatic ecosystems with neonicotinoids.

The decline of many populations of invertebrates, due mostly to the widespread presence of waterborne residues and the extreme chronic toxicity of neonicotinoids, is affecting the structure and function of aquatic ecosystems. Consequently, vertebrates that depend on insects and other aquatic invertebrates as their sole or main food resource are being affected. Declines of insectivore bird species are quite evident so far, but many other terrestrial and amphibian species may be at risk.

Solutions must be found soon if we are to save the biodiversity not only of aquatic ecosystems, but all other ecosystems linked by the food web. Since the prophylactic use of seeds treated with neonicotinoids is responsible for most of the soil and aquatic contamination, while there is evidence of little productivity gain, one obvious solution is to stop the marketing of seeds coated with these insecticides (van der Sluijs et al., 2015) and use alternative and carefully targeted methods for pest control in agriculture (Douglas and Tooker, 2015; Furlan and Kreutzweiser, 2015), such as integrated pest management (IPM). At the same time, remediation systems based on photolytic processes (Malato et al., 2001) and wetlands phyto-remediation (Beketov and Liess, 2008c) should be implemented to reduce as much as possible the current contamination by these and other pollutants.

AUTHOR CONTRIBUTIONS

FS wrote the first draft and KG and DH added and corrected the original version. All three authors contributed equally to the experimental data reviewed here.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2016.00071/full#supplementary-material

REFERENCES

Alexander, A. C., Culp, J. M., Liber, K., and Cessna, A. J. (2007). Effects of insecticide exposure on feeding inhibition in mayflies and oligochaetes. *Environ. Toxicol. Chem.* 26, 1726–1732. doi: 10.1897/07-015R.1

Alexander, A. C., Heard, K. S., and Culp, J. M. (2008). Emergent body size of mayfly survivors. *Freshw. Biol.* 53, 171–180. doi: 10.1111/j.1365-2427.2007.01880.x

Anderson, J. C., Dubetz, C., and Palace, V. P. (2015). Neonicotinoids in the Canadian aquatic environment: a literature review on current use products with a focus on fate, exposure, and biological effects. *Sci. Total Environ.* 505, 409–422. doi: 10.1016/j.scitotenv.2014.09.090

Bajeer, M. A., Nizamani, S. M., Sherazi, S. T. H., and Bhanger, M. I. (2012). Adsorption and leaching potential of imidacloprid pesticide through alluvial soil. *Am. J. Anal. Chem.* 3, 604–611. doi: 10.4236/ajac.2012.38079

Beketov, M. A., Kefford, B. J., Schäfer, R. B., and Liess, M. (2013). Pesticides reduce regional biodiversity of stream invertebrates. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11039–11043. doi: 10.1073/pnas.1305618110

Beketov, M. A., and Liess, M. (2008a). Acute and delayed effects of the neonicotinoid insecticide thiacloprid on seven freshwater arthropods. *Environ. Toxicol. Chem.* 27, 461–470. doi: 10.1897/07-322R.1

Beketov, M. A., and Liess, M. (2008b). Potential of 11 pesticides to initiate downstream drift of stream macroinvertebrates. *Arch. Environ. Contam. Toxicol.* 55, 247–253. doi: 10.1007/s00244-007-9104-3

Beketov, M. A., and Liess, M. (2008c). Variability of pesticide exposure in a stream mesocosm system: macrophyte-dominated vs. non-vegetated sections. *Environ. Pollut.* 156, 1364–1367. doi: 10.1016/j.envpol.2008.08.014

Beketov, M., Schäfer, R. B., Marwitz, A., Paschke, A., and Liess, M. (2008). Long-term stream invertebrate community alterations induced by the insecticide thiacloprid: effect concentrations and recovery dynamics. *Sci. Total Environ.* 405, 96–108. doi: 10.1016/j.scitotenv.2008.07.001

Bonmatin, J. M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C., et al. (2015). Environmental fate and exposure; neonicotinoids and fipronil. *Environ. Sci. Pollut. Res.* 22, 35–67. doi: 10.1007/s11356-014-3332-7

Böttger, R., Feibicke, M., Schaller, J., and Dudel, G. (2013). Effects of low-dosed imidacloprid pulses on the functional role of the caged amphipod *Gammarus roeseli* in stream mesocosms. *Ecotoxicol. Environ. Saf.* 93, 93–100. doi: 10.1016/j.ecoenv.2013.04.006

Brock, T. C. M., Belgers, J. D. M., Roessink, I., Cuppen, J. G. M., and Maund, S. J. (2010). Macroinvertebrate responses to insecticide application between sprayed and adjacent nonsprayed ditch sections of different sizes. *Environ. Toxicol. Chem.* 29, 1994–2008. doi: 10.1002/etc.238

Brown, A. W. A. (1978). *Ecology of Pesticides*. New York, NY: John Wiley & Sons, Inc.

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., et al. (2011). Patterns of widespread decline in North American bumble bees. *Proc. Natl. Acad. Sci. U.S.A.* 108, 662–667. doi: 10.1073/pnas.1014743108

Cataño, H. C., Carranza, E., Huamaní, C., and Hernández, A. F. (2008). Plasma cholinesterase levels and health symptoms in Peruvian farm workers exposed to organophosphate pesticides. *Arch. Environ. Contam. Toxicol.* 55, 153–159. doi: 10.1007/s00244-007-9095-0

CCME (2007). *Canadian Water Quality Guidelines: Imidacloprid. Scientific Supporting Document*. Winnipeg: Canadian Council of Ministers of the Environment.

Cleveland, C. B., Mayes, M. A., and Cryer, S. A. (2002). An ecological risk assessment for spinosad use on cotton. *Pest Manage. Sci.* 58, 70–84. doi: 10.1002/ps.424

Colombo, V., Mohr, S., Berghahn, R., and Pettigrove, V. J. (2013). Structural changes in a macrozoobenthos assemblage after imidacloprid pulses in aquatic field-based microcosms. *Arch. Environ. Contam. Toxicol.* 65, 683–692. doi: 10.1007/s00244-013-9940-2

Daam, M. A., Santos Pereira, A. C., Silva, E., Caetano, L., and Cerejeira, M. J. (2013). Preliminary aquatic risk assessment of imidacloprid after application in an experimental rice plot. *Ecotoxicol. Environ. Saf.* 97, 78–85. doi: 10.1016/j.ecoenv.2013.07.011

Denning, A., Ernst, W. R., Julien, G. R., Doe, K. G., Cook, A., Bernier, M., et al. (2004). *An Assessment of Buffer Zone Effectiveness in Reducing Pesticide Runoff from Potato Fields in Prince Edward Island (2001–2002)*. Atlantic Region: Environment Canada, Environmental Protection Branch.

Ding, Z., Yang, Y., Jin, H., Yu, H., Feng, J., Zhang, X., et al. (2004). Acute toxicity and bioconcentration factor of three pesticides on *Brachydanio rerio*. *Ying Yong Sheng Tai Xue Bao* 15, 888–890.

Douglas, M., and Tooker, J. F. (2015). Large-scale deployment of seed treatments has driven rapid increase in use of neonicotinoid insecticides and preemptive pest management in U.S. field crops. *Environ. Sci. Technol.* 49, 5088−5097. doi: 10.1021/es506141g

Druckrey, H., and Küpfmüller, K. (1949). *Dosis und Wirkung - Beiträge zur theoretischen Pharmakologie*. Freiburg im Breisgau: Cantor GmbH.

EFSA (2013). Conclusion on the peer review of the pesticide risk assessment for bees for the active substance imidacloprid. *EFSA J.* 11:3068. doi: 10.2903/j.efsa.2013.3068

Elbert, A., Haas, M., Springer, B., Thielert, W., and Nauen, R. (2008). Applied aspects of neonicotinoid uses in crop protection. *Pest Manag. Sci.* 64, 1099–1105. doi: 10.1002/ps.1616

Englert, D., Bundschuh, M., and Schulz, R. (2012). Thiacloprid affects trophic interaction between gammarids and mayflies. *Environ. Pollut.* 167, 41–46. doi: 10.1016/j.envpol.2012.03.024

Escher, B. I., Ashauer, R., Dyer, S., Hermens, J. L. M., Lee, J.-H., Leslie, H. A., et al. (2011). Crucial role of mechanisms and modes of toxic action for understanding tissue residue toxicity and internal effect concentrations of organic chemicals. *Integr. Environ. Assess. Manag.* 7, 28–49. doi: 10.1002/ieam.100

European Commission (2013). *Commission Implementing Regulation (EU) No 485/2013*. Brussels: Official Journal of the European Union.

Fitzpatrick, Ú., Murray, T. E., Paxton, R. J., Breen, J., Cotton, D., Santorum, V., et al. (2007). Rarity and decline in bumblebees - a test of causes and correlates in the Irish fauna. *Biol. Conserv.* 136, 185–194. doi: 10.1016/j.biocon.2006.11.012

Forister, M. L., Cousens, B., Harrison, J. G., Anderson, K., Thorne, J. H., Waetjen, D., et al. (2016). Increasing neonicotinoid use and the declining butterfly fauna of lowland California. *Biol. Lett.* 12:20160475. doi: 10.1098/rsbl.2016.0475

Fox, P. J., and Matthiessen, P. (1982). Acute toxicity to fish of low-dose aerosol applications of endosulfan to control tsetse fly in the Okavango delta, Botswana. *Environ. Polluti. Ser. A* 27, 129–142. doi: 10.1016/0143-1471(82)90105-2

Furlan, L., and Kreutzweiser, D. (2015). Alternatives to neonicotinoid insecticides for pest control: case studies in agriculture and forestry. *Environ. Sci. Pollut. Res.* 22, 135–147. doi: 10.1007/s11356-014-3628-7

Garric, A., and Hir, P. L. (2016). Loi sur la biodiversité: la France bannit les pesticides tueurs d'abeilles. *Le Monde*.

Gilburn, A. S., Bunnefeld, N., Wilson, J. M., Botham, M. S., Brereton, T. M., Fox, R., et al. (2015). Are neonicotinoid insecticides driving declines of widespread butterflies? *PeerJ* 3:e1402. doi: 10.7717/peerj.1402

González-Pradas, E., Ureía-Amate, M. D., Flores-Céspedes, F., Fernández-Pérez, M., Garratt, J., and Wilkins, R. (2002). Leaching of imidacloprid and procymidone in a greenhouse of southeast of Spain. *Soil Sci. Soc. Am. J.* 66, 1821–1828. doi: 10.2136/sssaj2002.1821

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol.* 50, 977–987. doi: 10.1111/1365-2664.12111

Hallmann, C. A., Foppen, R. P. B., van Turnhout, C. A. M., de Kroon, H., and Jongejans, E. (2014). Declines in insectivorous birds are associated with high neonicotinoid concentrations. *Nature* 511, 341–343. doi: 10.1038/nature13531

Hayasaka, D., Korenaga, T., Sánchez-Bayo, F., and Goka, K. (2012a). Differences in ecological impacts of systemic insecticides with different physicochemical properties on biocenosis of experimental paddy fields. *Ecotoxicology* 21, 191–201. doi: 10.1007/s10646-011-0778-y

Hayasaka, D., Korenaga, T., Suzuki, K., Saito, F., Sánchez-Bayo, F., and Goka, K. (2012b). Cumulative ecological impacts of two successive annual treatments of imidacloprid and fipronil on aquatic communities of paddy mesocosms. *Ecotoxicol. Environ. Saf.* 80, 355–362. doi: 10.1016/j.ecoenv.2012.04.004

Hayasaka, D., Suzuki, K., Nomura, T., Nishiyama, M., Nagai, T., Sánchez-Bayo, F., et al. (2013). Comparison of acute toxicity of two neonicotinoid insecticides, imidacloprid and clothianidin, to five cladoceran species. *J. Pestic. Sci.* 38, 44–47. doi: 10.1584/jpestics.D12-061

Hladik, M. L., and Calhoun, D. L. (2012). *Analysis of the Herbicide Diuron, Three Diuron Degradates, and Six Neonicotinoid Insecticides in Water—Method Details and Application to Two Georgia streams*. Reston, VA: U.S. Geological Survey.

Hladik, M. L., Kolpin, D. W., and Kuivila, K. M. (2014). Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. *Environ. Pollut.* 193, 189–196. doi: 10.1016/j.envpol.2014.06.033

Jeschke, P., and Nauen, R. (2008). Neonicotinoids—from zero to hero in insecticide chemistry. *Pest Manag. Sci.* 64, 1084–1098. doi: 10.1002/ps.1631

Jeschke, P., Nauen, R., Schindler, M., and Elbert, A. (2011). Overview of the status and global strategy for neonicotinoids. *J. Agric. Food Chem.* 59, 2897–2908. doi: 10.1021/jf101303g

Jinguji, H., Thuyet, D., Ueda, T., and Watanabe, H. (2013). Effect of imidacloprid and fipronil pesticide application on *Sympetrum infuscatum* (Libellulidae: Odonata) larvae and adults. *Paddy Water Environ.* 11, 277–284. doi: 10.1007/s10333-012-0317-3

Johnson, J. D., and Pettis, J. S. (2014). A survey of imidacloprid levels in water sources potentially frequented by honeybees (*Apis mellifera*) in the Eastern USA. *Water Air Soil Pollut.* 225, 1–6. doi: 10.1007/s11270-014-2127-2

Jones, A., Harrington, P., and Turnbull, G. (2014). Neonicotinoid concentrations in arable soils after seed treatment applications in preceding years. *Pest Manag. Sci.* 70, 1780–1784. doi: 10.1002/ps.3836

Kattwinkel, M., Reichert, P., Rüegg, J., Liess, M., and Schuwirth, N. (2016). Modeling macroinvertebrate community dynamics in stream mesocosms contaminated with a pesticide. *Environ. Sci. Technol.* 50, 3165–3173. doi: 10.1021/acs.est.5b04068

Krebs, J. R., Wilson, J. D., Bradbury, R. B., and Siriwardena, G. M. (1999). The second Silent Spring? *Nature* 400, 611–612. doi: 10.1038/23127

Kreuger, J., Graaf, S., Patring, J., and Adielsson, S. (2010). *Pesticides in Surface Water in Areas with Open Ground and Greenhouse Horticultural Crops in Sweden 2008*. Uppsala: Swedish University of Agricultural Sciences.

Kreutzweiser, D., Good, K., Chartrand, D., Scarr, T., and Thompson, D. (2007). Non-target effects on aquatic decomposer organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. *Ecotoxicol. Environ. Saf.* 68, 315–325. doi: 10.1016/j.ecoenv.2007.04.011

Kreutzweiser, D. P., Good, K. P., Chartrand, D. T., Scarr, T. A., Holmes, S. B., and Thompson, D. G. (2008a). Effects on litter-dwelling earthworms and microbial decomposition of soil-applied imidacloprid for control of wood-boring insects. *Pest Manag. Sci.* 64, 112–118. doi: 10.1002/ps.1478

Kreutzweiser, D. P., Good, K. P., Chartrand, D. T., Scarr, T. A., and Thompson, D. G. (2008b). Toxicity of the systemic insecticide, imidacloprid, to forest stream insects and microbial communities. *Bull. Environ. Contam. Toxicol.* 80, 211–214. doi: 10.1007/s00128-007-9347-8

Kunce, W., Josefsson, S., Örberg, J., and Johansson, F. (2015). Combination effects of pyrethroids and neonicotinoids on development and survival of *Chironomus riparius*. *Ecotoxicol. Environ. Saf.* 122, 426–431. doi: 10.1016/j.ecoenv.2015.09.008

Kurwadkar, S., Wheat, R., McGahan, D. G., and Mitchell, F. (2014). Evaluation of leaching potential of three systemic neonicotinoid insecticides in vineyard soil. *J. Contam. Hydrol.* 170, 86–94. doi: 10.1016/j.jconhyd.2014.09.009

Kwok, Y. C., Hsieh, D. P. H., and Wong, P. K. (2005). Toxicity identification evaluation (TIE) of pore water of contaminated marine sediments collected from Hong Kong waters. *Mar. Pollut. Bull.* 51, 1085–1091. doi: 10.1016/j.marpolbul.2005.06.009

Lahr, J., Diallo, A. O., Gadji, B., Diouf, P. S., Bedeaux, J. J. M., Badji, A., et al. (2000). Ecological effects of experimental insecticide applications on invertebrates in Sahelian temporary ponds. *Environ. Toxicol. Chem.* 19, 1278–1289. doi: 10.1002/etc.5620190509

Liess, M., Foit, K., Becker, A., Hassold, E., Dolciotti, I., Kattwinkel, M., et al. (2013). Culmination of low-dose pesticide effects. *Environ. Sci. Technol.* 47, 8862–8868. doi: 10.1021/es401346d

Liu, X., Xu, X., Li, C., Zhang, H., Fu, Q., Shao, X., et al. (2015). Degradation of chiral neonicotinoid insecticide cycloxaprid in flooded and anoxic soil. *Chemosphere* 119, 334–341. doi: 10.1016/j.chemosphere.2014.06.016

Macfadyen, S., Hardie, D. C., Fagan, L., Stefanova, K., Perry, K. D., DeGraaf, H. E., et al. (2014). Reducing insecticide use in broad-acre grains production: an Australian study. *PLoS ONE* 9:e89119. doi: 10.1371/journal.pone.0089119

Malato, S., Caceres, J., Agüera, A., Mezcua, M., Hernando, D., Vial, J., et al. (2001). Degradation of imidacloprid in water by photo-Fenton and TiO_2 photocatalysis at a solar pilot plant: a comparative study. *Environ. Sci. Technol.* 35, 4359–4366. doi: 10.1021/es000289k

Mason, R., Tennekes, H., Sánchez-Bayo, F., and Jepsen, P. U. (2013). Immune suppression by neonicotinoid insecticides at the root of global wildlife declines. *J. Environ. Immunol. Toxicol.* 1, 3–12. doi: 10.7178/jeit.1

Matsumura, F. (1985). *Toxicology of Pesticides*. New York, NY: Plenum Press.

Matthiessen, P., Fox, P. J., Douthwaite, R. J., and Wood, A. B. (1982). Accumulation of endosulfan residues in fish and their predators after aerial spraying for the control of tsetse fly in Botswana. *Pestic. Sci.* 13, 39–48. doi: 10.1002/ps.2780130107

Maund, S. J., Sherratt, T. N., Stickland, T., Biggs, J., Williams, P., Shillabeer, N., et al. (1997). Ecological considerations in pesticide risk assessment for aquatic ecosystems. *Pestic. Sci.* 49, 185–190.

Medina, M., Barata, C., Telfer, T., and Baird, D. J. (2004). Effects of cypermethrin on marine plankton communities: a simulated field study using mesocosms. *Ecotoxicol. Environ. Saf.* 58, 236–245. doi: 10.1016/j.ecoenv.2003.07.001

Miranda, G. R. B., Raetano, C. G., Silva, E., Daam, M. A., and Cerejeira, M. J. (2011). Environmental fate of neonicotinoids and classification of their potential risks to hypogean, epygean, and surface water ecosystems in Brazil. *Hum. Ecol. Risk Assess.* 17, 981–995. doi: 10.1080/10807039.2011.588159

Morrissey, C. A., Mineau, P., Devries, J. H., Sánchez-Bayo, F., Liess, M., Cavallaro, M. C., et al. (2015). Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. *Environ. Int.* 74, 291–303. doi: 10.1016/j.envint.2014.10.024

Morse, J. C., Stark, B. P., and McCafferty, P. W. (1993). Southern Appalachian streams at risk: implications for mayflies, stoneflies, caddisflies, and other aquatic biota. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 3, 293–303. doi: 10.1002/aqc.3270030404

Motobayashi, T., Genka, M., Khanh Phong, T., and Watanabe, H. (2012). Effects of formulation and treatment method of imidacloprid in nursery boxes on aquatic insects inhabiting rice paddy fields. *Jpn. J. Appl. Entomol. Zool.* 56, 169–172. doi: 10.1303/jjaez.2012.169

Phong, T. K., Nhung, D. T. T., Motobayashi, T., Thuyet, D. Q., and Watanabe, H. (2009). Fate and transport of nursery-box-applied tricyclazole and imidacloprid in paddy fields. *Water Air Soil Pollut.* 202, 3–12. doi: 10.1007/s11270-008-9953-z

Nyman, A.-M., Hintermeister, A., Schirmer, K., and Ashauer, R. (2013). The insecticide imidacloprid causes mortality of the freshwater amphipod *Gammarus pulex* by interfering with feeding behavior. *PLoS ONE* 8:e62472. doi: 10.1371/journal.pone.0062472

Osborne, J. L. (2012). Ecology: bumblebees and pesticides. *Nature* 491, 43–45. doi: 10.1038/nature11637

Parsons, J. T., and Surgeoner, G. A. (1991). Effect of exposure time on the acute toxicities of permethrin, fenitrothion, carbaryl and carbofuran to mosquito larvae. *Environ. Toxicol. Chem.* 10, 1219–1227. doi: 10.1002/etc.5620100913

Pestana, J. L. T., Alexander, A. C., Culp, J. M., Baird, D. J., Cessna, A. J., and Soares, A. M. V. M. (2009). Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. *Environ. Pollut.* 157, 2328–2334. doi: 10.1016/j.envpol.2009.03.027

Peveling, R., McWilliam, A. N., Nagel, P., Rasolomanana, H., Raholijaona, Rakotomianina, L., et al. (2003). Impact of locust control on harvester termites and endemic vertebrate predators in Madagascar. *J. Appl. Ecol.* 40, 729–741. doi: 10.1046/j.1365-2664.2003.00833.x

Phillips, P. J., and Bode, R. W. (2004). Pesticides in surface water runoff in south-eastern New York State, USA: seasonal and stormflow effects on concentrations. *Pest Manag. Sci.* 60, 531–543. doi: 10.1002/ps.879

Potts, G. R. (1986). *The Partridge - Pesticides, Predation and Conservation*. London: Collins.

Roessink, I., Merga, L. B., Zweers, H. J., and van den Brink, P. J. (2013). The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. *Environ. Toxicol. Chem.* 32, 1096–1100. doi: 10.1002/etc.2201

Rondeau, G., Sánchez-Bayo, F., Tennekes, H. A., Decourtye, A., Ramírez-Romero, R., and Desneux, N. (2014). Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. *Sci. Rep.* 4:5566. doi: 10.1038/srep05566

Sánchez-Bayo, F. (1991). "Temporal diversity and structure within a bird community," in *Diversidad Biologica/Biological Diversity*, eds F. D. Pineda, M.

A. Casado, J. M. D. Miguel, and J. Montalvo (Madrid: Centro de Estudios Ramon Areces S.A.), 155–159.

Sánchez-Bayo, F. (2009). From simple toxicological models to prediction of toxic effects in time. *Ecotoxicology* 18, 343–354. doi: 10.1007/s10646-008-0290-1

Sánchez-Bayo, F. (2011). "Impacts of agricultural pesticides on terrestrial ecosystems," in *Ecological Impacts of Toxic Chemicals*, eds F. Sánchez-Bayo, P. J. van den Brink, and R. Mann (Beijing: Bentham Science Publishers), 63–87. Available online at: http://www.benthamdirect.com/51436/volume/1

Sánchez-Bayo, F. (2014). The trouble with neonicotinoids. *Science* 346, 806–807. doi: 10.1126/science.1259159

Sánchez-Bayo, F., Ahmad, R., and Goka, K. (2007). "Evaluation of the standard quotient and EcoRR methodologies based on field monitoring from rice fields," in *Rational Environmental Management of Agrochemicals*, eds I. R. Kennedy, K. R. Solomon, S. J. Gee, A. N. Crossan, S. Wang, and F. Sánchez-Bayo (Washington, DC: American Chemical Society), 66–86.

Sánchez-Bayo, F., and Goka, K. (2005). Unexpected effects of zinc pyrithione and imidacloprid on Japanese medaka fish (*Oryzias latipes*). *Aquat. Toxicol.* 74, 285–293. doi: 10.1016/j.aquatox.2005.06.003

Sánchez-Bayo, F., and Goka, K. (2006a). Ecological effects of the insecticide imidacloprid and a pollutant from antidandruff shampoo in experimental rice fields. *Environ. Toxicol. Chem.* 25, 1677–1687. doi: 10.1897/05-404R.1

Sánchez-Bayo, F., and Goka, K. (2006b). Influence of light in acute toxicity bioassays of imidacloprid and zinc pyrithione to zooplankton crustaceans *Aquat. Toxicol.* 78, 262–271. doi: 10.1016/j.aquatox.2006.03.009

Sánchez-Bayo, F., and Hyne, R. V. (2014). Detection and analysis of neonicotinoids in river waters - development of a passive sampler for three commonly used insecticides. *Chemosphere* 99, 143–151. doi: 10.1016/j.chemosphere.2013.10.051

Schaafsma, A., Limay-Rios, V., Baute, T., Smith, J., and Xue, Y. (2015). Neonicotinoid insecticide residues in surface water and soil associated with commercial maize (corn) fields in southwestern Ontario. *PLoS ONE* 10:e0118139. doi: 10.1371/journal.pone.0118139

Schaafsma, A., Limay-Rios, V., Xue, Y., Smith, J., and Baute, T. (2016). Field-scale examination of neonicotinoid insecticide persistence in soil as a result of seed treatment use in commercial maize (corn) fields in southwestern Ontario. *Environ. Toxicol. Chem.* 35, 295–302. doi: 10.1002/etc.3231

Seagraves, M. P., and Lundgren, J. G. (2012). Effects of neonicitinoid seed treatments on soybean aphid and its natural enemies. *J. Pest Sci.* 85, 125–132. doi: 10.1007/s10340-011-0374-1

Siebers, J., Binner, R., and Wittich, K.-P. (2003). Investigation on downwind short-range transport of pesticides after application in agricultural crops. *Chemosphere* 51, 397–407. doi: 10.1016/S0045-6535(02)00820-2

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Chagnon, M., Downs, C., et al. (2015). Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environ. Sci. Pollut. Res.* 22, 5–34. doi: 10.1007/s11356-014-3470-y

Smit, C. E., Posthuma-Doodeman, C. J. A. M., van Vlaardingen, P. L. A., and de Jong, F. M. W. (2015). Ecotoxicity of imidacloprid to aquatic organisms: derivation of water quality standards for peak and long-term exposure. *Hum. Ecol. Risk Assess.* 21, 1608–1630. doi: 10.1080/10807039.2014.964071

Song, M. Y., Stark, J. D., and Brown, J. J. (1997). Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. *Environ. Toxicol. Chem.* 16, 2494–2500. doi: 10.1002/etc.5620161209

Sorg, M., Schwan, H., Stenmans, W., and MüLler, A. (2013). Ermittlung der Biomassen flugaktiver Insekten im Naturschutzgebiet Orbroicher Bruch mit Malaise Fallen in den Jahren 1989 und 2013. *Proc. Krefeld Entomol. Soc.* 1, 1–5.

Sorrell, B. K., and Boon, P. I. (1992). Biogeochemistry of billabong sediments. II Seasonal variations in methane production. *Freshw. Biol.* 27, 435–445. doi: 10.1111/j.1365-2427.1992.tb00552.x

Starner, K., and Goh, K. (2012). Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010–2011. *Bull. Environ. Contam. Toxicol.* 88, 316–321. doi: 10.1007/s00128-011-0515-5

Stoughton, S. J., Liber, K., Culp, J., and Cessna, A. (2008). Acute and chronic toxicity of imidacloprid to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca* under constant- and pulse-exposure conditions. *Arch. Environ. Contam. Toxicol.* 54, 662–673. doi: 10.1007/s00244-007-9073-6

Sur, R., and Stork, A. (2003). Uptake, translocation and metabolism of imidacloprid in plants. *Bull. Insectol.* 56, 35–40.

Suter, G. W. II, and Cormier, S. M. (2015). Why care about aquatic insects: uses, benefits, and services. *Integr. Environ. Assess. Manag.* 11, 188–194. doi: 10.1002/ieam.1600

Tennekes, H. A. (2010a). The significance of the Druckrey-Küpfmüller equation for risk assessment - the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. *Toxicology* 276, 1–4. doi: 10.1016/j.tox.2010.07.005

Tennekes, H. A. (2010b). *The Systemic Insecticides: A Disaster in the Making*. Zutphen: ETS Nederland BV.

Tennekes, H. A., and Sánchez-Bayo, F. (2013). The molecular basis of simple relationships between exposure concentration and toxic effects with time. *Toxicology* 309, 39–51. doi: 10.1016/j.tox.2013.04.007

Thuyet, D. Q., Jorgenson, B. C., Wissel-Tyson, C., Watanabe, H., and Young, T. M. (2012). Wash off of imidacloprid and fipronil from turf and concrete surfaces using simulated rainfall. *Sci. Total Environ.* 414, 515–524. doi: 10.1016/j.scitotenv.2011.10.051

Thuyet, D. Q., Watanabe, H., Yamazaki, K., and Takagi, K. (2011). Photodegradation of imidacloprid and fipronil in rice–paddy water. *Bull. Environ. Contam. Toxicol.* 86, 548–553. doi: 10.1007/s00128-011-0243-x

Tomizawa, M., and Casida, J. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.* 45, 247–268. doi: 10.1146/annurev.pharmtox.45.120403.095930

Tomlin, C. D. S. (ed.). (2009). *The e-Pesticide Manual*, *12th Edn*. Surrey: British Crop Protection Council.

Uğurlu, P., Ünlü, E., and Satar, E. I. (2015). The toxicological effects of thiamethoxam on *Gammarus kischineffensis* (Schellenberg 1937) (Crustacea: Amphipoda). *Environ. Toxicol. Pharmacol.* 39, 720–726. doi: 10.1016/j.etap.2015.01.013

USGS (2002). *Concentrations of Pesticides and Pesticide Degradates in the Croton River Water- Shed in Southeastern New York, July–September 2000*. New York, NY: United States Geological Survey.

van den Brink, P. J., Smeden, J. M. V., Bekele, R. S., Dierick, W., De Gelder, D. D., Noteboom, M., et al. (2016). Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. *Environ. Toxicol. Chem.* 35, 128–133. doi: 10.1002/etc.3152

van den Brink, P. J., Wijngaarden, R. P. A. V., Lucassen, W. G. H., Brock, T. C. M., and Leeuwangh, P. (1996). Effects of the insecticide Dursban 4E (active ingredient chlorpyrifos) in outdoor experimental ditches: II. Invertebrate community responses and recovery. *Environ. Toxicol. Chem.* 15, 1143–1153.

van der Sluijs, J. P., Amaral-Rogers, V., Belzunces, L. P., Lexmond, M. F. I. J. B. V., Bonmatin, J.-M., Chagnon, M., et al. (2015). Conclusions of the Worldwide Integrated Assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. *Environ. Sci. Pollut. Res.* 22, 148–154. doi: 10.1007/s11356-014-3229-5

van der Sluijs, J. P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.-M., and Belzunces, L. P. (2013). Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr. Opin. Environ. Sustain.* 5, 293–305. doi: 10.1016/j.cosust.2013.05.007

van Dijk, T. C., van Staalduinen, M. A., and van der Sluijs, J. P. (2013). Macro-invertebrate decline in surface water polluted with imidacloprid. *PLoS ONE* 8:e62374. doi: 10.1371/journal.pone.0062374

Vehovszky, Á., Farkas, A., Ács, A., Stoliar, O., Székács, A., Mörtl, M., et al. (2015). Neonicotinoid insecticides inhibit cholinergic neurotransmission in a molluscan (*Lymnaea stagnalis*) nervous system. *Aquat. Toxicol.* 167, 172–179. doi: 10.1016/j.aquatox.2015.08.009

Vijver, M. G., and van den Brink, P. J. (2014). Macro-invertebrate decline in surface water polluted with imidacloprid: a rebuttal and some new analyses. *PLoS ONE* 9:e89837. doi: 10.1371/journal.pone.0089837

von der Ohe, P. C., Prüss, A., Schäfer, R. B., Liess, M., de Deckere, E., and Brack, W. (2007). Water quality indices across Europe - a comparison of the good ecological status of five river basins. *J. Environ. Monit.* 9, 970–978. doi: 10.1039/b704699p

Weston, D. P., Chen, D., and Lydy, M. J. (2015). Stormwater-related transport of the insecticides bifenthrin, fipronil, imidacloprid, and chlorpyrifos into a tidal

wetland, San Francisco Bay, California. *Sci. Total Environ.* 527–528, 18–25. doi: 10.1016/j.scitotenv.2015.04.095

Woodcock, B. A., Isaac, N. J. B., Bullock, J. M., Roy, D. B., Garthwaite, D. G., Crowe, A., et al. (2016). Impacts of neonicotinoid use on long-term population changes in wild bees in England. *Nat. Commun.* 7:12459. doi: 10.1038/ncomms12459

Yamamoto, A., Terao, T., Hisatomi, H., Kawasaki, H., and Arakawa, R. (2012). Evaluation of river pollution of neonicotinoids in Osaka City (Japan) by LC/MS with dopant-assisted photoionisation. *J. Environ. Monit.* 14, 2189–2194. doi: 10.1039/c2em30296a

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Sánchez-Bayo, Goka and Hayasaka. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 21 February 2017
doi: 10.3389/fpls.2017.00215

Single and Combined Effects of Pesticide Seed Dressings and Herbicides on Earthworms, Soil Microorganisms, and Litter Decomposition

*Willem Van Hoesel[1], Alexandra Tiefenbacher[1], Nina König[1], Verena M. Dorn[1], Julia F. Hagenguth[1], Urša Prah[1], Theresia Widhalm[1], Viktoria Wiklicky[1], Robert Koller[2,3], Michael Bonkowski[2], Jan Lagerlöf[4], Andreas Ratzenböck[5] and Johann G. Zaller[1]**

[1] Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Life Sciences Vienna, Vienna, Austria, [2] Department of Terrestrial Ecology, Institute of Zoology, University of Cologne, Cologne, Germany, [3] Institute of Bio- and Geosciences, IBG-2: Plant Sciences, Forschungszentrum Jülich, Jülich, Germany, [4] Department of Ecology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, [5] Austrian Agency for Health and Food Safety GmbH (AGES), Vienna, Austria

OPEN ACCESS

Edited by:
Vimala Nair,
University of Florida, USA

Reviewed by:
Andrey S. Zaitsev,
Justus-Liebig-University, Germany/
Severtsov Institute of Ecology and Evolution (RAS), Russia
Astrid Rita Taylor,
Swedish University of Agricultural Sciences, Sweden

***Correspondence:**
Johann G. Zaller
johann.zaller@boku.ac.at

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Plant Science

Received: *26 September 2016*
Accepted: *06 February 2017*
Published: *21 February 2017*

Citation:
Van Hoesel W, Tiefenbacher A, König N, Dorn VM, Hagenguth JF, Prah U, Widhalm T, Wiklicky V, Koller R, Bonkowski M, Lagerlöf J, Ratzenböck A and Zaller JG (2017) Single and Combined Effects of Pesticide Seed Dressings and Herbicides on Earthworms, Soil Microorganisms, and Litter Decomposition. Front. Plant Sci. 8:215. doi: 10.3389/fpls.2017.00215

Seed dressing, i.e., the treatment of crop seeds with insecticides and/or fungicides, aiming to protect seeds from pests and diseases, is widely used in conventional agriculture. During the growing season, those crop fields often receive additional broadband herbicide applications. However, despite this broad utilization, very little is known on potential side effects or interactions between these different pesticide classes on soil organisms. In a greenhouse pot experiment, we studied single and interactive effects of seed dressing of winter wheat (*Triticum aestivum* L. *var. Capo*) with neonicotinoid insecticides and/or strobilurin and triazolinthione fungicides and an additional one-time application of a glyphosate-based herbicide on the activity of earthworms, soil microorganisms, litter decomposition, and crop growth. To further address food-web interactions, earthworms were introduced to half of the experimental units as an additional experimental factor. Seed dressings significantly reduced the surface activity of earthworms with no difference whether insecticides or fungicides were used. Moreover, seed dressing effects on earthworm activity were intensified by herbicides (significant herbicide × seed dressing interaction). Neither seed dressings nor herbicide application affected litter decomposition, soil basal respiration, microbial biomass, or specific respiration. Seed dressing did also not affect wheat growth. We conclude that interactive effects on soil biota and processes of different pesticide classes should receive more attention in ecotoxicological research.

Keywords: agrochemicals, agroecology, neonicotinoids, non-target effects, pesticide, seed coatings, soil organisms, glyphosate-herbicide

INTRODUCTION

The prophylactic treatment of crop seeds with insecticides and/or fungicides, so called "seed dressing," is very common in conventional agriculture, especially for wheat, oilseed rape, sugar beet, and maize (Krupinsky et al., 2002; Elbert et al., 2008). Many of the agrochemicals used for seed dressings act systemically, meaning that they will be distributed across the whole crop plant and potentially also released into the soil. Recently, neonicotinoid insecticides used for seed dressings have received increased attention because of their proved harm to insect pollinators (Gill et al., 2012; Whitehorn et al., 2012; Easton and Goulson, 2013; Pisa et al., 2015). Besides insecticides, various classes of fungicides are used for seed dressings. However, very little is known about their potential non-target effects. Due to the persistence of neonicotinoid pesticides in the soil of up to several years, non-target effects have been observed in various soil organisms inhabiting agroecosystems (Goulson, 2013; Köhler and Triebskorn, 2013; Chagnon et al., 2015; Pisa et al., 2015). Reports on effects of insecticide and fungicide seed dressings vary from stimulating Collembola surface activity (Zaller et al., 2016) to reducing collembolan reproduction (Alves et al., 2014), increasing numbers of protozoa and reducing plant decomposition rate (Zaller et al., 2016) to increasing earthworm mortality (Alves et al., 2013) or not influencing earthworm activity (Zaller et al., 2016).

Fields where dressed seeds are applied are often additionally treated with glyphosate-based herbicides, e.g., for pre-harvest desiccation (Carvalho et al., 2009). Although for decades this group of herbicides has been considered to be without harm toward non-target soil organisms, scientific evidence is mounting for adverse effects on symbiotic mycorrhizal fungi (Druille et al., 2013; Zaller et al., 2014), earthworms (Dalby et al., 1995; Morowati, 2000; Yasmin and D'souza, 2007; Piola et al., 2013; Pelosi et al., 2014; Gaupp-Berghausen et al., 2015), and soil microbial communities (Zabaloy et al., 2012; Imparato et al., 2016). However, the combined effects of different pesticide classes on soil organisms have received little attention (Yasmin and D'souza, 2007; Santos et al., 2011; Van Der Sluijs et al., 2015). Moreover, we are not aware of any study targeting the interactive effects of seed dressings and glyphosate-based herbicides on soil organisms and soil processes.

Microorganisms and soil fauna contribute to the decomposition of plant residues in agricultural fields, the mineralization of plant residues, and the recycling of plant nutrients (Berg, 2009; Paul, 2015). The soil macrofauna, especially vertically-burrowing earthworms, translocate plant material and plant seeds from the soil surface into deeper soil layers (Zaller and Saxler, 2007; Eisenhauer et al., 2008), creating hotspots of high microbial activity in deeper soil layers. Additionally, earthworms also feed on saprophytic fungi and other microorganisms driving decomposition (Scheu and Setälä, 2002; Curry and Schmidt, 2007). The resulting rate of litter decomposition will thus be an integrated effect of both earthworm and/or microbial activity (Hättenschwiler et al., 2005).

The aim of this study was to assess (i) to what extent pesticide seed dressings affect earthworm activity, soil microorganisms and litter decomposition, (ii) the existence of potential interactive effects between seed dressings and a glyphosate-herbicide application (i.e., cocktail effects) on these soil functional groups, and (iii) whether the presense of earthworms alters potential pesticide effects. We hypothesized, that insecticide and fungicide seed dressings would indirectly affect earthworms by reducing their microbial food sources. Fungicide seed dressing should exert negative effects on the fungal component of the soil microbial biomass (Merrington et al., 2002), while glyphosate-based herbicide application is assumed to decrease the overall activity of soil microorganisms (Sannino and Gianfreda, 2001; Zaller et al., 2014) and earthworms (Gaupp-Berghausen et al., 2015) and to increase respiration as a stress response of sensitive species (Zabaloy et al., 2012). Lastly, we expected non-target effects of two pesticide classes to be more severe than single applications.

MATERIALS AND METHODS

Experimental Design

The experiment consisted of a full-factorial design including the factors Seed dressing (SD, 3 levels), Earthworms (EW, 2 levels), Herbicide application (Herbic, 2 levels), and their interactions; see below for details.

Experimental Factors: Seed Dressings, Earthworms, and Herbicide Application

We tested the effects of three types of seed dressings in this experiment: no seed dressing (treatment NO), seed dressing dominated by neonicotinoid insecticide and associated fungicides (treatment Insectic) and a fungicide seed dressing (treatment Fungic; **Table 1**). Each seed dressing treatment was replicated 10 times.

Eight days after the seeding two adult specimens of *Lumbricus terrestris* L. per mesocosm were added to half of the mesocosms (total average earthworm fresh mass added across treatments: 7.5 ± 0.8 g mesocosm^{-1}; treatment +EW); no earthworms were added to the other half of the mesocosms (treatment –EW). Each earthworm treatment was replicated 5 times.

Thirty-one days after seeding, a broadband glyphosate-based herbicide (Roundup Lb Plus; Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany) was applied to half of the mesocosms (treatment +Herbic); no herbicide was applied to the other half of the mesocosms (treatment –Herbic). This resulted in five replicates of each of the above mentioned treatments after this stage.

Experimental Setup

The experiment was conducted in an experimental greenhouse at the University of Natural Resources and Life Sciences Vienna (BOKU), Austria (N48°14′12.4, E16°20′08.4). The 60 cylindrical mesocosms (diameter: 25 cm, height: 60 cm, volume: 30 l) were randomly placed in three double-rows each consisting of 2 × 10 mesocosms in east-west direction. The mesocosms were filled with a soil mixture consisting of a substrate

TABLE 1 | Overview of the seed dressing treatments and glyphosate-based herbicide used in the current experiment.

Treatment/Brand name	Active ingredient	Pesticide class	Chemical class	Conc. (g l^{-1})	Systemic?
INSECTICIDE SEED DRESSING					
Gaucho 600FS edigo	Imidacloprid	Insecticide	Neonicotinoid	600	Yes
	Prothioconazole	Fungicide	Triazolinthione	100	Yes
Celest Extra 050FS	Difenoconazole	Fungicide	Conazole	25	No
	Fludioxonil	Fungicide	Pyrrole	25	No
FUNGICIDE SEED DRESSING					
EfA Universal	Fluoxastrobin	Fungicide	Strobilurin	75	No
	Prothioconazole	Fungicide	Triazolinthione	50	Yes
	Fluopyram	Fungicide	Pyridylethylamide	10	No
	Tebuconazole	Fungicide	Triazole	7.5	No
G/YPHOSATE-BASED HERBICIDE					
Roundup Lb Plus	Glyphosate	Herbicide	Organophosphate	360	Yes

mixture of 75% vol/vol haplic chernozem from an arable field of the BOKU Research Farm (Groß Enzersdorf, Austria) that was mixed with 1.4–2.2 mm quartz sand (general soil characteristics are: C:N ratio 17.15, pH 7.45 ± 0.02) and 25% commercial peat-free potting soil containing bark humus, wood fibers, and green waste compost, sand, and mineral (NPK) fertilizer. No soil sterilization was performed. All mesocosms were outfitted with a 20 cm high barrier of plastic sheet glued at the top of the mesocosm in order to prevent any organisms from escaping. Between October and December 2013, these mesocosms were used to test non-target effects of seed dressings on earthworms and Collembola and the soil decomposition processes by microorganisms (Zaller et al., 2016). After the termination of the previous experiment, mesocosms were kept in the greenhouse, watering was stopped and heating was kept at 20°C in order to induce a complete dry-out and defaunation of the soil. After 3 months, careful examinations did not show any signs of earthworm or Collembola activity in the pots. For the current experiment, the original treatments were retained: i.e., seed dressings and earthworm treatments were assigned to the same mesocosms than in the former experiment. The Collembola treatment of the former experiment was excluded in the current experiment; no Collembola activity was observed during the course of the current study.

Each mesocosm was sown with 18 seeds treated with pesticide seed dressings (**Table 1**) of winter wheat (*Triticum aestivum* L. *var. Capo*) placed in 1 cm depth in a consistent pattern. Seeding density corresponded to 367 seeds m^{-2} that is within the recommended seeding density for this variety. Seed material with these dressings is available for Austrian farmers and was provided by the Austrian Agency for Health and Food Security (AGES, Vienna, Austria).

Added earthworms were purchased at a local fishing equipment shop in Vienna (www.anglertreff.at). Earthworms were placed on the soil surface and buried themselves into the soil within several minutes. During the experiment, all mesocosms received 1 g of dried hay per week, placed at the top of the soil; the mesocosms that did not contain earthworms received the same amount of hay to ensure equal nutrient input.

Thirty-one days after seeding, Roundup Lb Plus was applied to wheat plants that were about 12 cm high at that time. Roundup Lb Plus contains 30.8% glyphosate as active ingredient; 486 g l^{-1} as isopropylamin salt (**Table 1**). This formulation is registered for use in arable crops, forestry, horticulture, viticulture, and private use (http://pmg.ages.at/export/PMG/PMG/web/reg/3393_901.html). We applied the herbicide as recommended on the manual of the spray bottle, so that all plants were covered with a mist. This resulted in a total of 1.47 ml m^{-2} that is 1.47 times the recommended application amount of 1 ml m^{-2} of this product. Mesocosms near the treated ones were protected by a plastic sheet. Plant death due to the herbicide application was observed about 7 days after spraying.

The current experiment lasted from March until June 2014, covering 97 days. The average air temperature during this period was 18 ± 2.4°C (mean ± standard deviation) at a relative humidity of 59.4 ± 29.5%; measured with data loggers placed 2 m above the greenhouse floor (Tinytag, Gemini Data Loggers, UK).

Measurements

Earthworm Activity, Earthworm Survival, and Development

In order to assess surface activity of earthworms, the toothpick method was used (Zaller et al., 2014). Briefly, 12 regular wooden toothpicks (length: 6.5 cm) were randomly inserted vertically across the surface, with the tips slightly stuck in the ground. Earthworms foraging aboveground knock over or incline the toothpicks; the number of toothpicks differing from their original upright position thus indicates the above ground activity of earthworms. Toothpicks were inserted in the evening and assessed the following morning; this was regularly done once a week resulting in five assessments before herbicide application, and twice a week after the herbicide application (3–4 day interval) resulting in 16 assessments. Toothpicks were removed between sampling dates. For the analysis of earthworm activity three different categories of disturbance of the toothpicks were used: value 0.1 for slight disturbance, 0.5 for disturbance in which the toothpick was tilted more than or around 45° and 1 for those that were found horizontally on the surface. The number of the toothpicks within each category was multiplied

with the category value and then summed and taken as an index measure of aboveground earthworm activity. Because we were interested to see if either the size or the number of earthworms is more responsive to our pesticide treatments, activity of earthworms was further expressed as number of toothpicks moved per g earthworm biomass (specific earthworm activity) and per number of earthworms (individual earthworm activity). After wheat harvesting, mesocosms were turned over and each mesocosm searched for earthworms by two persons for 8 min. Earthworms were counted, washed free of attached soil, carefully dried off on a paper towel, and collectively weighed per mesocosm.

Soil Microbial Biomass and Activity

At the end of the experiment (97 days after seeding) five random soil samples per mesocosm were taken with a soil corer (diameter 1 cm, depth 5 cm) for the analysis of soil basal respiration, microbial biomass, and specific respiration. These samples were stored in polypropylene plastic bags, cooled at 5°C and express-mailed to the soil laboratory of the Department for Terrestrial Ecology at the University of Cologne, Germany, for analysis. Soil microbial biomass (C_{mic}) was determined from a 3 g subsample of fresh soil samples. Microbial biomass was measured by substrate-induced respiration (Anderson, 1978) using an automated respirometer based on electrolytic O_2 micro compensation (Scheu, 1992), as outlined in Beck et al. (1997). For basal respiration, the average O_2 consumption rate of samples not amended with glucose was measured during 15–20 h after attachment of samples to the respirometer. Microbial specific respiration (qO_2, $\mu l\ O_2\ \mu g^{-1}\ C_{mic}\ h^{-1}$) was calculated as the quotient between basal respiration and microbial biomass.

Litter Decomposition

Litter decomposition rate (k) and stabilization factor (S) were assessed using the tea bag method (Keuskamp et al., 2013) to assess the breakdown of labile of easily degradable and recalcitrant organic matter. In every mesocosm, we inserted 2 tea bags containing rooibos tea (Lipton, EAN: 87 22700 18843 8) and 2 containing green tea (Lipton, EAN: 87 22700 05552 5) at 8 cm depth. The tea bags were dried for 2 days at 55°C and weighed before insertion into the soil and were left in the soil for 84 days. Afterwards tea bags were excavated, cleaned from adhered soil particles, and dried for 3 days at 55°C and weighed. The decomposition rate (k) and the stabilization factor (S) was calculated after Keuskamp et al. (2013) considering the hydrolysable fraction of 0.842 g g^{-1} for green tea and of 0.552 g g^{-1} for rooibos tea. Green tea and rooibos tea have different decomposition rates meaning that rooibos tea decomposes slower and continues when labile material in green tea has already been consumed. The stabilization process begins during the decomposition of the labile fraction of organic material (Prescott, 2010). This method was already used in some other studies to examine non-target effects of glyphosate-based herbicides (Gaupp-Berghausen et al., 2015) and insecticide and fungicide seed dressings (Zaller et al., 2016).

In each mesocosm, soil moisture was measured using time domain reflectometry (Trime Pico 63/32 probe; IMKO, Micromodultechnik GmbH, Ettlingen, Germany). These measurements were taken once a week by inserting the 20 cm long probe in the center of each mesocosm.

Wheat Growth and Biomass Production

Wheat height was recorded once a week on all plants per mesocosm by measuring their height from the soil surface to the tip of the longest leaf. Height measurement was stopped in mesocosms after the herbicide was applied on day 31 after seeding. On day 43 of the experiment, above ground biomass from all the mesocosms was harvested by cutting all wheat plants at the soil surface using scissors, then dried for 48 h at 55°C and weighed. The plant density per mesocosm on the day of herbicide spraying was on average 16.3 ± 1.3 plants $mesocosm^{-1}$ and at the moment of final harvest of the remaining mesocosms 16.4 ± 1.1 plants $mesocosm^{-1}$.

Statistical Analyses

All variables were tested for normality using P-P plots and homogeneity of variances using the Levene test and log transformed when necessary. Influence of seed dressing (SD) or herbicide application (Herbic) on earthworm surface activity (average moved toothpicks pot^{-1} day^{-1}, cumulated moved toothpicks, activity per earthworm biomass) was tested using repeated measures analysis of variance (ANOVA). When Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, the Greenhouse-Geisser correction was used. Three factorial analysis of variance (ANOVA) with the factors SD, Herbic or Earthworms and interactive effects among SD × Herbic, SD × Earthworms or Herbic × Earthworms was included in the statistical model to examine effects on litter decomposition, soil basal respiration, qCO_2, microbial biomass, wheat growth, and wheat biomass as well as on earthworm numbers and biomass at the end of the experiment. In all analyses soil moisture content was used as a covariate. Statistical analyses were carried out using Minitab statistical software (Release 14, Minitab Corp., PA, USA).

RESULTS

Earthworm surface activity (per g earthworm, per number of earthworms, and cumulated surface activity) was significantly reduced by seed dressings compared to undressed seeds, regardless whether insecticide or fungicide seed dressings were used (**Figure 1**, **Table 2**). Across seed dressings, herbicide application reduced specific earthworm activity; while individual earthworm activity was affected by an interaction between seed dressings and herbicide application (**Figure 1**, **Table 2**). In our experiment, seed dressings reduced (cumulative) earthworm activity by 9.2% while herbicide application reduced it by 19.3%.

At the end of the experiment we found 88.3% of the initially inserted adult earthworms and 59.6% of the initially inserted biomass of earthworms (**Table 3**). Neither the number, nor the biomass of retrieved adult earthworms at harvest was affected by seed dressings, herbicide applications or their interactions (adult earthworm numbers: *SD* – $F = 1.142$, $P = 0.345$; Herbic – $F = 0.111$, $P = 0.744$; SD × Herbic – $F = 0.331$,

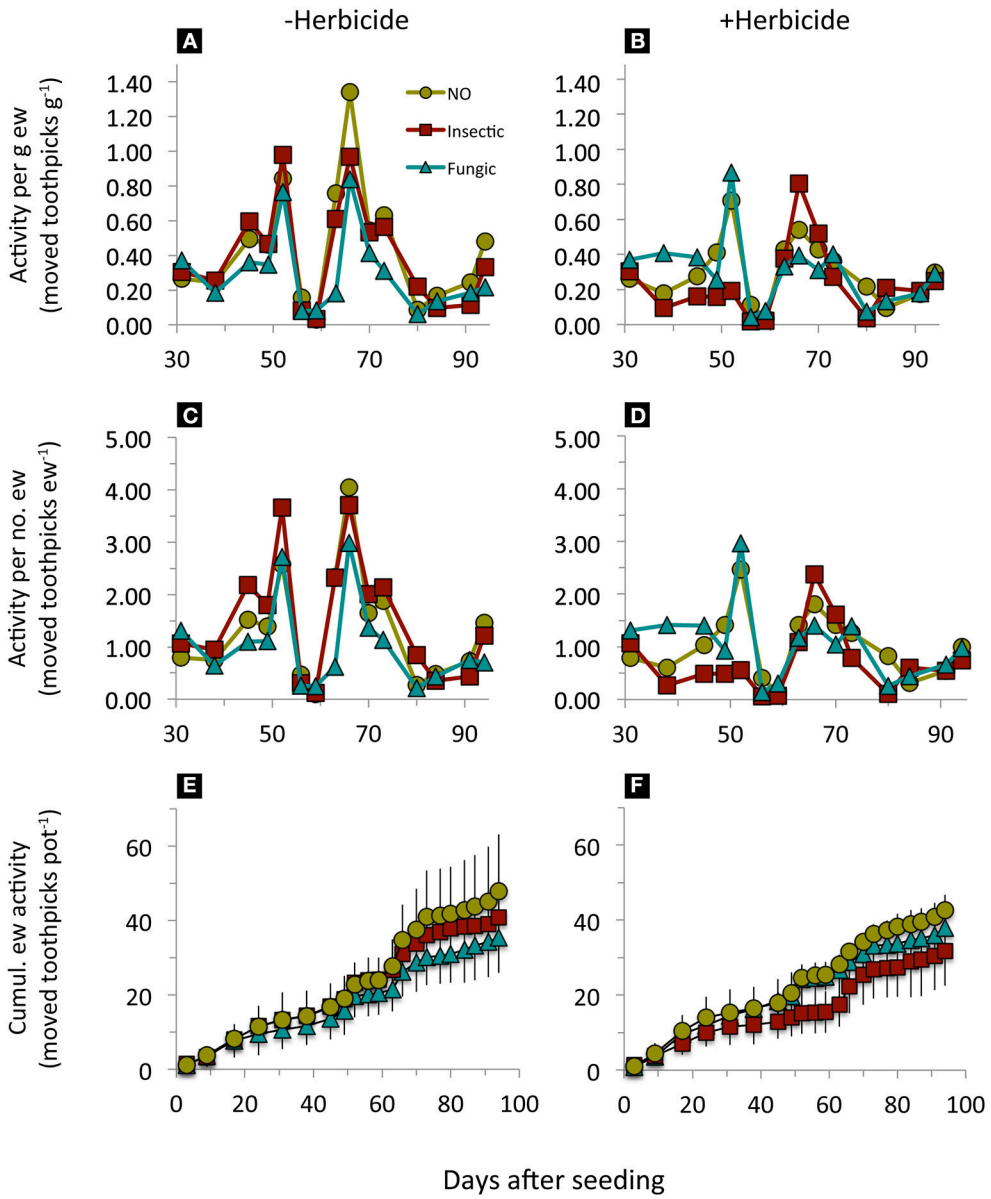


FIGURE 1 | Earthworm surface activity in mesocosms where winter wheat with different pesticide seed dressings was sown (NO, no seed dressing; insectic: neonicotinoid insecticide seed dressing; fungic: fungicide seed dressing) without (A,C,E) or with glyphosate-based herbicide application (B,D,F) at day 31. Means, $n = 5$. Statistical results in **Table 2**.

$P = 0.723$; adult earthworm biomass: SD – $F = 1.352$, $P = 0.288$; Herbic – $F = 0.088$, $P = 0.770$; $SD \times$ Herbic – $F = 0.937$, $P = 0.414$). Moreover, at the end of the experiment, all mesocosms contained many juvenile, initially not inserted earthworms. Mesocosms initially without earthworm inoculation contained at harvest 42.47 ± 19.02 juvenile individuals with 0.85 ± 0.41 g mesocosm^{-1}, mesocosms with earthworm inoculation contained 42.17 ± 21.18 juvenile individuals with 5.46 ± 4.46 g mesocosm^{-1}. Total earthworm numbers retrieved at harvest was neither affected by earthworm treatment ($F = 0.583$, $P = 0.449$), seed dressing ($F = 1.249$, $P = 0.296$), herbicide application ($F = 0.722$, $P = 0.400$) nor the interactions of these factors. Total biomass of earthworms at harvest was significantly lower in non-earthworm treatments ($F = 33.253$, $P < 0.001$) but not affected by seed dressing ($F = 0.525$, $P = 0.595$), herbicide ($F = 0.713$, $P = 0.403$) or their interactions.

Litter decomposition rate (k) and stabilization factor (S) decreased when added *L. terrestris* earthworms were present, but were not affected by seed dressings, herbicide application or their interactions (**Table 2**, **Figure 2**). Soil basal respiration, microbial biomass, and specific respiration were neither affected by seed

TABLE 2 | Statistical results testing the effects of seed dressings (SD), earthworms (EW) and glyphosate-herbicide application on earthworm surface activity, litter decomposition, soil microbial activity, wheat growth, and wheat biomass production in mesocosms.

Parameter	Seed Dressing (SD)		Earthworms (EW)		Herbicide (Herbic)		*SD* × EW		*SD* × Herbic		EW × Herbic	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
EW surf. act., specific (toothp. g^{-1} EW)^{rmA}	4.349	**0.025**	n.a.	n.a.	4.44	**0.046**	n.a.	n.a.	2.872	0.077	n.a.	n.a.
EW surf. act., specific (toothp. no^{-1} EW)^{rmA}	2.776	0.083	n.a.	n.a.	3.571	0.071	n.a.	n.a.	4.769	**0.019**	n.a.	n.a.
EW surf. act., mean (toothpicks)^{rmA}	4.011	**0.033**	n.a.	n.a.	2.619	0.120	n.a.	n.a.	0.440	0.649	n.a.	n.a.
EW cumul. surf. act. (toothpicks)^{rmA}	3.742	**0.040**	n.a.	n.a.	0.116	0.736	n.a.	n.a.	2.422	0.112	n.a.	n.a.
Litter decomposition rate (k)	1.856	0.168	14.987	**<0.001**	0.001	0.971	0.252	0.092	1.159	0.323	0.416	0.522
Litter stabilization index (S)	1.410	0.254	14.463	**<0.001**	0.005	0.942	2.399	0.102	0.658	0.522	1.527	0.223
Soil basal respiration (1-Jg COr C g^{-1} h-1)	0.525	0.595	0.02	0.889	0.428	0.516	1.651	0.203	0.279	0.758	0.132	0.718
Soil microbial biomass (Cmic, I-Jg C g- 1)	1.527	0.228	1.522	0.224	0.183	0.671	0.032	0.969	1.356	0.268	0.046	0.831
Soil qC02 (µg $C0_2$–C g- h^{-1} Cmic h^{-1})	0.880	0.164	0.2	0.657	1.069	0.307	0.751	0.478	2.036	0.142	0.219	0.642
Wheat height (cm)^{rmA}	1.843	0.181	9.843	**0.005**	n.a.	n.a.	1.214	0.315	n.a.	n.a.	n.a.	n.a.
Wheat biomass (g)	0.668	0.517	4.925	**0.032**	n.a.	n.a.	0.377	0.688	n.a.	n.a.	n.a.	n.a.

Significant effects in bold.
n.a. Not applicable; ^{rmA} analyzed with repeated measures ANOVA.

TABLE 3 | Earthworm numbers and biomass (fresh mass) retrieved from mesocosms where winter wheat with seed dressings was sown or glyphosate-herbicide was applied.

Experimental factors		Adult earthworms (*L. terrestris*)		Juvenile unidentified, earthworms	
Seed dressing	Herbicide	Number	Biomass (g)	Number	Biomass (g)
NO	No	1.6 ± 1.5	3.2 ± 3.6	38 ± 26	0.607 ± 0.411
	Yes	2.0 ± 1.2	5.4 ± 3.3	45 ± 21	1.008 ± 0.647
Insecticide	No	1.0 ± 1.4	3.0 ± 4.2	41 ± 18	0.840 ± 0.441
	Yes	2.0 ± 1.2	4.6 ± 3.8	38 ± 19	0.697 ± 0.399
Fungicide	No	2.2 ± 2.6	5.7 ± 7.2	45 ± 17	0.874 ± 0.329
	Yes	1.8 ± 2.2	4.9 ± 5.4	38 ± 26	0.918 ± 0.822

Means ± st. dev., n = 10 for factor seed dressing; n = 5 when glyphosate was applied. No significant effects of seed dressings or herbicide application on earthworm numbers or biomass were observed.

dressings, earthworm, herbicide application, or their interactions (**Table 2**, **Figure 3**).

Wheat height and biomass production until 3 days before herbicide application were significantly reduced by earthworm activity but not affected by seed dressings nor an interaction between earthworms and seed dressings (**Table 2**, **Figure 4**).

DISCUSSION

To the best of our knowledge, this study is among the first studies addressing single and combined effects of different pesticide classes on soil organisms and soil processes. We aimed to mimic a typical farmland situation: wheat sown with pesticide treated seeds receiving an additional herbicide application later in the season. Our findings showed that seed dressings reduced earthworm activity regardless which pesticide class was used for seed treatment. Herbicide application itself reduced, and in interaction with seed dressings further decreased earthworm activity. Activity of soil microorganisms or litter decomposition appeared to be little affected by these pesticides.

Seed Dressing Effects

The current study is an expansion of a previous one where the effect of seed dressings were studied on the activity of earthworms, Collembola, and soil microorganisms (Zaller et al., 2016). In the current experiment we additionally applied a herbicide treatment in order to test a common farmland situation. Our current findings are partly in contrast with our previous findings where no effects of seed dressings on earthworm activity and a reduced litter decomposition in response to seed dressings was observed (Zaller et al., 2016). We attribute the different outcomes to the following reasons. First, in the previous study seed dressings were applied for the first time, while in the current experiment by utilizing the mesocosms from the previous experiment, seed dressings were applied for a second time within 5 months. Studies have shown that pesticides from seed dressing application (at least with neonicotinoid insecticides) accumulate in soils which could have resulted in an increased impact on non-target organisms (Goulson, 2013). Second, the response of soil microorganisms and litter decomposition after a one-time application of pesticides (Zaller et al., 2016) suggest an initial sensitivity but a rapid adaptation of soil microorganisms to metabolize these substances (Griffiths et al., 2001; Liu et al., 2011; Cycon et al., 2013). Similar to the previous study, we found no effect of seed dressings on crop

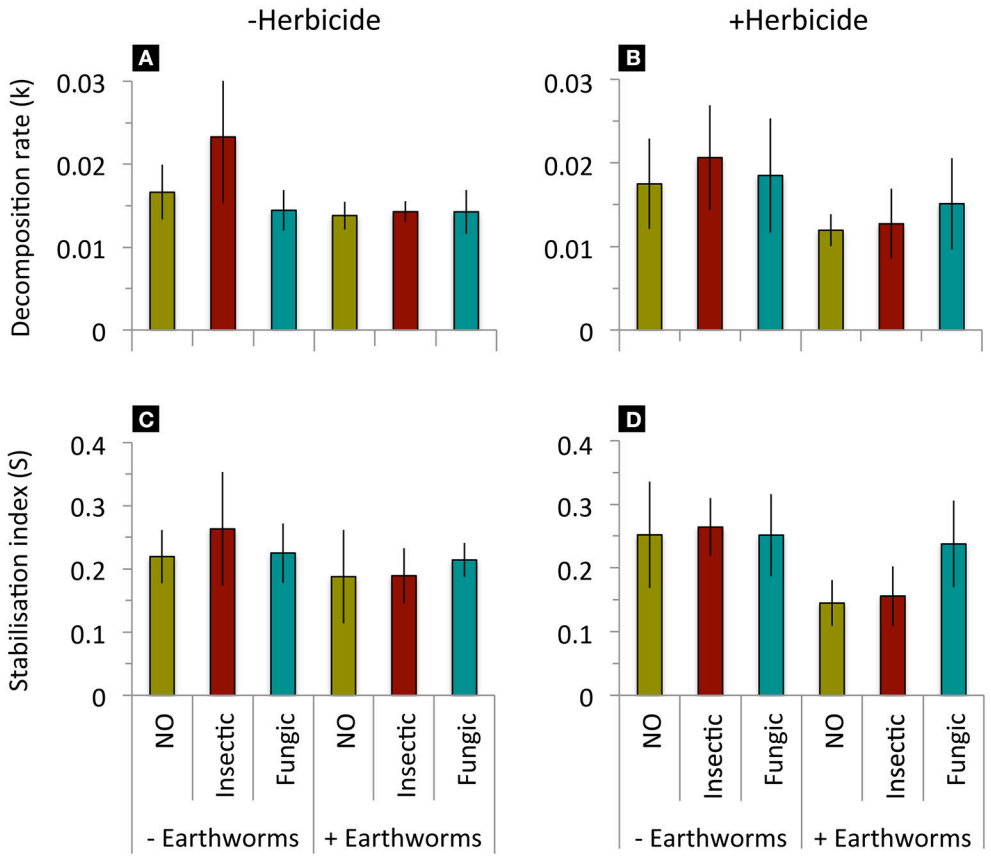


FIGURE 2 | Litter decomposition decomposition rate (k) and stabilization factor (S) in mesocosms without or with addition of *L. terrestris* earthworms, without (A,C) or with (B,D) glyphosate-based herbicide application. Means ± st. dev., $n = 10$. Statistical results in **Table 2**.

growth or biomass production. This might be due to a reduced pressure of pest insects or fungal diseases in our greenhouse experiment. However, even under field conditions the effect of seed dressing on yields appears to be negligible (Goulson, 2013; Budge et al., 2015; Furlan and Kreutzweiser, 2015).

There is good evidence that neonicotinoid insecticides directly affect earthworms (Dittbrenner et al., 2010, 2011). However, while we investigated actual pesticide formulations used by farmers, others investigated effects of the direct active ingredients. Significant loss in body mass of *L. terrestris* was observed with imidacloprid at concentrations ranging between 0.66 and 4.00 mg kg^{-1} soil after only 7–14 days of exposure (Dittbrenner et al., 2010, 2011). These sub-lethal effects occur well below the Predicted Environmental Concentration range of 0.33–0.66 mg kg^{-1} soil (Dittbrenner et al., 2010). Most likely, overall pesticide concentrations in soil were much lower in the current study with addition of only 16 treated seeds per mesocosm, however no data are available of the concentrations in the soils in the mesocosms used. We assume that earthworms perhaps also came in direct contact with the pesticides by feeding on the treated seeds (Milcu et al., 2006; Zaller and Saxler, 2007; Forey et al., 2011).

For other earthworm species than used in the current study, neonicotinoid insecticides resulted in an avoidance of treated soils (Dittbrenner et al., 2011, 2012), an altered burrowing activity (Capowiez et al., 2003, 2006; Capowiez and Bérard, 2006), DNA damage (Zang et al., 2000) or increased mortality (Tu et al., 2011). When comparing the acute toxicity of 24 insecticides on the earthworm species *E. fetida*, the neonicotinoid imidacloprid was listed in the category super toxic in both contact filter paper and soil toxicity bioassay tests (Wang et al., 2012a,b). However, earthworm responses to pesticides have been shown to be species-specific and the reaction of one species precludes a serious assessment across all earthworms (Pelosi et al., 2014; Pisa et al., 2015). Earthworm species also differ in their response to different pesticide classes: species feeding on the soil surface, are more affected by pesticides applied aboveground than those feeding deeper in the soil (Pelosi et al., 2014). Besides insecticides also fungicides had detrimental effects on earthworms (Jänsch et al., 2006). However, in comparison with herbicide and fungicides, insecticides show a more negative effect on three earthworm species (*Allobophora chlorotica, Lumbricus castaneus, L. terrestris)* (Pelosi et al., 2013).

Studies testing non-target effects of fungicide classes used in our seed dressings are very rare. A triazole fungicide application

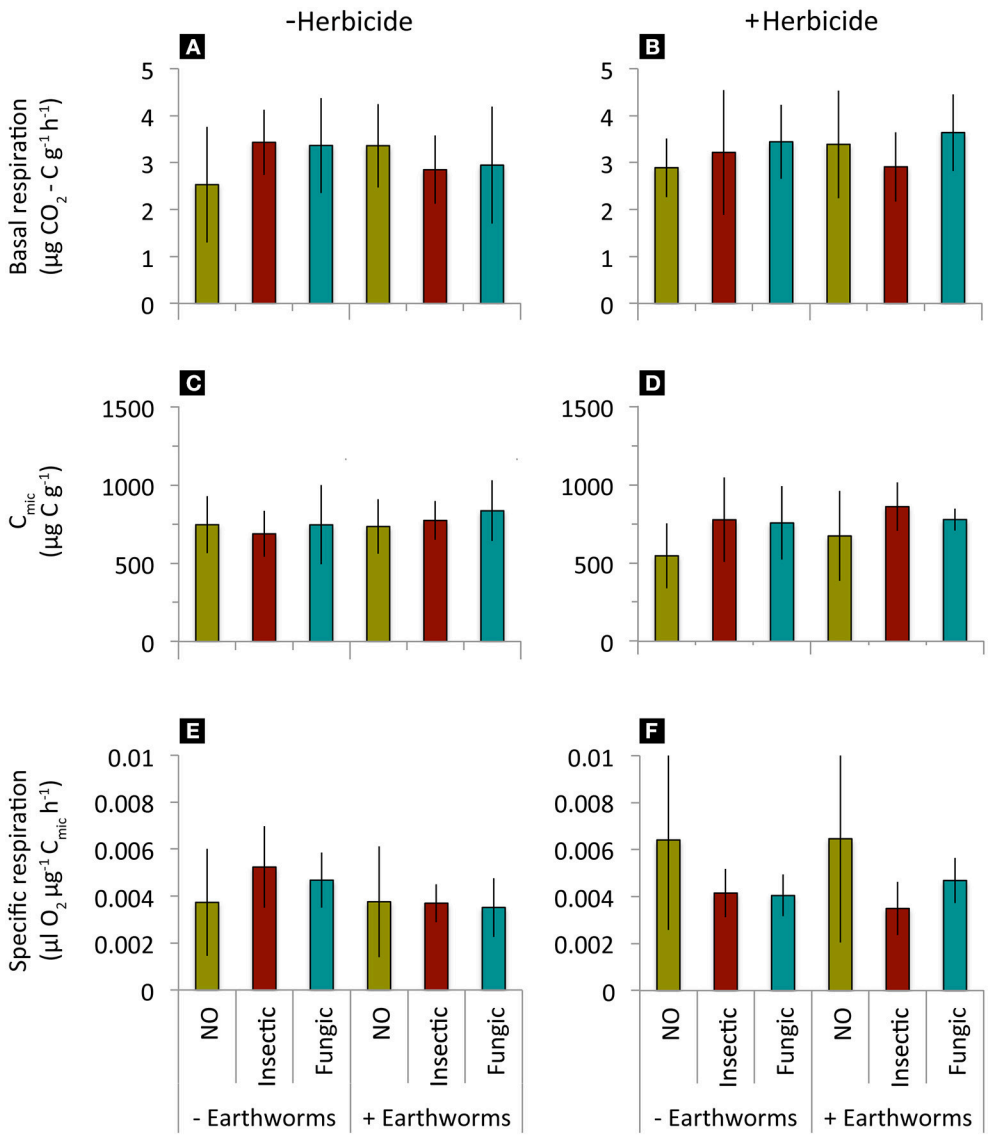


FIGURE 3 | Soil basal respiration, microbial biomass and specific respiration in mesocosms where winter wheat treated with different seed dressings (NO, no seed dressing; Insectic, neonicotinoid insecticide seed dressing; Fungic, fungicide seed dressing) were sown, without or with addition of *L. terrestris* earthworms and without (A,C,E) or with (B,D,F) glyphosate-herbicide application. Means ± st. dev., $n = 10$. Statistical results in **Table 2**.

resulted in a negative impact on the epidermic cells of *E. fetida* earthworms (Gao et al., 2013), however it is unclear whether there was a similar mode of action in our earthworm species. Clearly, there is a great demand for more studies in this subject.

Herbicide Effects

The reduction in earthworm activity after application of glyphosate-based herbicide is in accordance with recent findings studying the same earthworm species (Gaupp-Berghausen et al., 2015) although a lower dosage was used in the current experiment. After the herbicide application, the seed dressings further reduced earthworm activity, indicating possible synergistic effects of these different pesticide classes. In other studies a glyphosate-based herbicide also reduced reproduction (Casabe et al., 2007; Gaupp-Berghausen et al., 2015) and led to decreased growth and survival (Eijsackers et al., 2005). Another study shows that glyphosate herbicide application resulted in a high percentage (50%) of lethargic *Lumbricus* sp., while the combined effect with a pesticide resulted in increased mortality (Green et al., 2008). Even though we did not specifically investigate the reproduction of *L. terrestris* in the current experiment, the high numbers of juvenile, not identifiable earthworm species in each mesocosm indicated that hatching rates from cocoons were neither compromised by seed dressings nor the herbicide application.

When reporting non-target effects of pesticide formulations it is important to also consider side effects of numerous not-declared surfactants in these formulations as they might be more

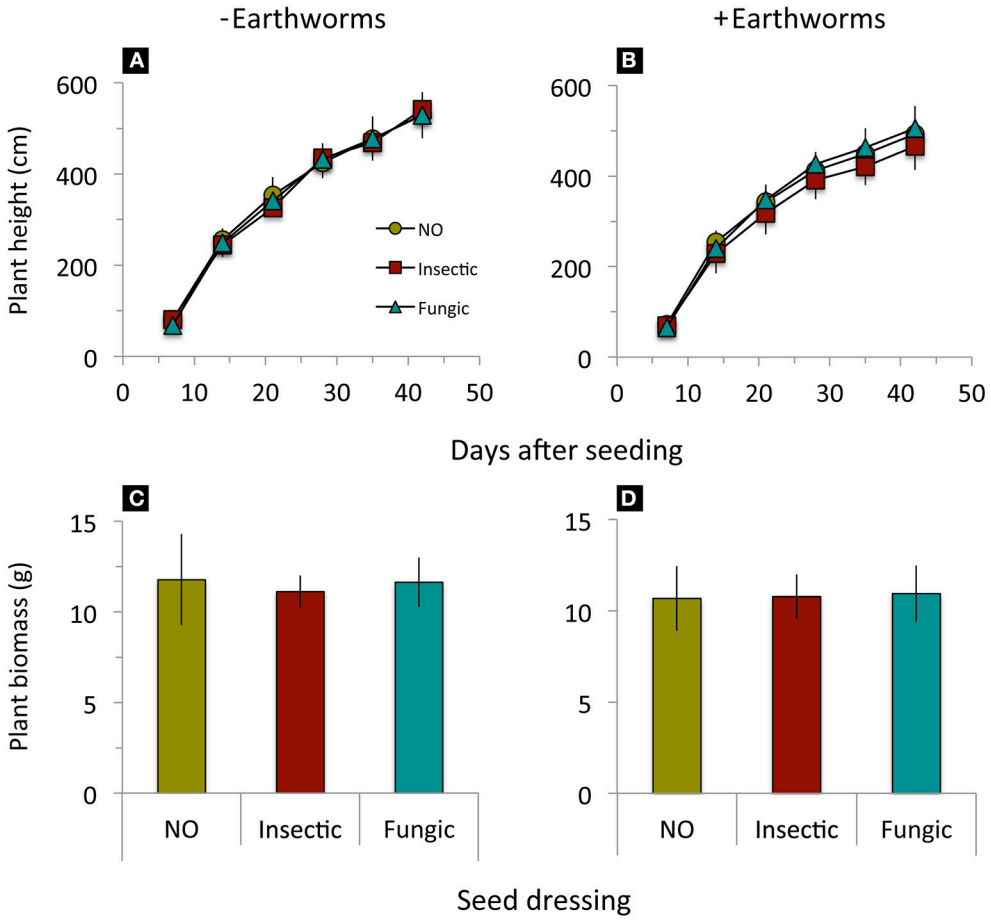


FIGURE 4 | Height growth and shoot biomass production of winter wheat treated with different seed dressings (NO, no seed dressing; Insectic, neonicotinoid insecticide seed dressing; Fungic, fungicide seed dressing) grown in mesocosms without (A,C) or with (B,D) addition of *L. terrestris* earthworms. Means ± st. dev., $n = 10$. Statistical results in **Table 2**.

toxic than the active ingredient itself (Moore et al., 2012; Cuhra et al., 2016; Mullin et al., 2016).

Earthworm Effects

Earthworms did not interact with seed dressings or herbicide application. This is contrast to previous studies (Zaller et al., 2014, 2016; Gaupp-Berghausen et al., 2015) but might just reflect specific responses of earthworms to different pesticides. Our understanding on feedback relations between pesticides, species interactions, populations and communities is very limited and demands more detailed studies (Köhler and Triebskorn, 2013). Contrary to our expectations, earthworms reduced litter decomposition, and reduced wheat growth and biomass production. A reduction in litter decomposition by earthworms was most likely an indirect effect via physical alterations of the soil environment as the organic matter in litter bags was only accessible to soil micro- and meso-fauna but not to earthworms. Such indirect effects could result from earthworm grazing on soil fungi and microorganisms as well as on soil meso- and micro-fauna (Edwards and Fletcher, 1988; Curry and Schmidt, 2007) thereby reducing overall decomposition. Earthworms are generally considered to increase plant growth (Van Groenigen et al., 2014), but not in all situations (Zaller and Arnone, 1999) and also reduced plant growth in presence of earthworms has been observed (Zaller et al., 2013; Arnone and Zaller, 2014). However, the influence of earthworms on plant growth and biomass production, and to which direction, depend mainly on earthworm and plant species in the system (Laossi et al., 2010; Doan et al., 2013) and still much is unknown about the precise earthworm-plant relationships (Scheu, 2003) or earthworm effects on root production (Arnone and Zaller, 2014).

CONCLUSIONS

The current findings in addition to our previous ones (Zaller et al., 2016) suggest different sensitivity of soil organisms dependent on how often pesticide treated seeds were sown. We found that micro- and meso-fauna were already influenced after a single seed dressing application, while macro-fauna responded only after the second seed dressing application in the current study. It is unclear whether this is a more widespread phenomenon because ecotoxicological tests very rarely investigate repeated applications of pesticides (Pelosi et al., 2014). To what extent pesticide-induced community

tolerance is responsible for acute vs. chronic toxicity of pesticides on earthworms is another underrepresented research area. In the current study we observed for the first time interactive effects on soil organisms between pesticides in seed dressings and surface applied herbicides. This indicates that pesticide risk assessments considering a single species subjected to a one time application of one pesticide class might underestimate the real world situation in agricultural fields.

AUTHOR CONTRIBUTIONS

JGZ, WVH conceived and designed the experiment; WVH, AT, NK, VD, JH, UP, TW, VW conducted the experiment; WVH, RK, MB, JL, AR, JGZ analyzed the data; all authors jointly wrote the manuscript.

FUNDING

This study was partly funded by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management.

ACKNOWLEDGMENTS

We would like to thank Yoko Muraoka, Edith Gruber and Norbert Schuller for help in the greenhouse. We are grateful to the reviewers for improvements on earlier versions of this manuscript.

REFERENCES

Alves, P. R., Cardoso, E., Martines, A. M., Sousa, J. P., and Pasini, A. (2013). Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions. *Chemosphere* 90, 2674–2682. doi: 10.1016/j.chemosphere.2012.11.046

Alves, P. R., Cardoso, E., Martines, A. M., Sousa, J. P., and Pasini, A. (2014). Seed dressing pesticides on springtails in two ecotoxicological laboratory tests. *Ecotoxicol. Environ. Saf.* 105, 65–71. doi: 10.1016/j.ecoenv.2014.04.010

Anderson, J. M. (1978). A method to quantify soil-microhabitat complexity and its application to a study of soil animal species diversity. *Soil Biol. Biochem.* 10, 77–78. doi: 10.1016/0038-0717(78)90014-7

Arnone, J. A., and Zaller, J. G. (2014). Earthworm effects on native grassland root system dynamics under natural and increased rainfall. *Front. Plant Sci.* 5:152. doi: 10.3389/fpls.2014.00152

Beck, T., Joergensen, R. G., Kandeler, E., Makeschin, F., Nuss, E., Oberholzer, H. R., et al. (1997). An inter-laboratory comparison of ten different ways of measuring soil microbial biomass C. *Soil Biol. Biochem.* 29, 1023–1032. doi: 10.1016/S0038-0717(97)00030-8

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Budge, G. E., Garthwaite, D., Crowe, A., Boatman, N. D., Delaplane, K. S., Brown, M. A., et al. (2015). Evidence for pollinator cost and farming benefits of neonicotinoid seed coatings on oilseed rape. *Sci. Rep.* 5:12574. doi: 10.1038/srep12574

Capowiez, Y., Bastardie, F., and Costagliola, G. (2006). Sublethal effects of imidacloprid on the burrowing behaviour of two earthworm species: modifications of the 3D burrow systems in artificial cores and consequences on gas diffusion in soil. *Soil Biol. Biochem.* 38, 285–293. doi: 10.1016/j.soilbio.2005.05.014

Capowiez, Y., and Bérard, A. (2006). Assessment of the effects of imidacloprid on the behavior of two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*) using 2D terraria. *Ecotoxicol. Environ. Saf.* 64, 198–206. doi: 10.1016/j.ecoenv.2005.02.013

Capowiez, Y., Rault, M., Mazzia, C., and Belzunces, L. (2003). Earthworm behaviour as a biomarker - a case study using imidacloprid. *Pedobiologia* 47, 542–547. doi: 10.1078/0031-4056-00226

Carvalho, S. J. P., Damin, V., Dias, A. C. R., Melo, M. S. C., Nicolai, M., and Christoffoleti, P. J. (2009). Weed desiccation with glyphosate mixed with urea or ammonium sulfate. *Planta Daninha* 27, 353–361. doi: 10.1590/S0100-83582009000200019

Casabe, N., Piola, L., Fuchs, J., Oneto, M. A. L., Pamparato, L., Kesten, E. et al. (2007). Ecotoxicological assessment of the effects of glyphosate and chlorpyrifos in an Argentine soya field. *J. Soils Sediments* 8, 1–8. doi: 10.1065/jss2007.04.224

Chagnon, M., Kreutzweiser, D., Mitchell, E. A. D., Morrissey, C. A., Noome, D. A., and Van Der Sluijs, J. P. (2015). Risks of large-scale use of systemic insecticides to ecosystem functioning and services. *Environ. Sci. Pollut. Res.* 22, 119–134. doi: 10.1007/s11356-014-3277-x

Cuhra, M., Bøhn, T., and Cuhra, P. (2016). Glyphosate: too much of a good thing? *Front. Environ. Sci.* 4:28. doi: 10.3389/fenvs.2016.00028

Curry, J. P., and Schmidt, O. (2007). The feeding ecology of earthworms - a review. *Pedobiologia* 50, 463–477. doi: 10.1016/j.pedobi.2006.09.001

Cycon, M., Markowicz, A., Borymski, S., Wójcik, M., and Piotrowska-Seget, Z. (2013). Imidacloprid induces changes in the structure, genetic diversity and catabolic activity of soil microbial communities. *J. Environ. Manage.* 131, 55–65. doi: 10.1016/j.jenvman.2013.09.041

Dalby, P. R., Baker, G. H., and Smith, S. E. (1995). Glyphosate, 2,4-DB and dimethoate: effects on earthworm survival and growth. *Soil Biol. Biochem.* 27, 1661–1662. doi: 10.1016/0038-0717(95)00091-R

Dittbrenner, N., Capowiez, Y., Kohler, H. R., and Triebskorn, R. (2012). Stress protein response (Hsp70) and avoidance behaviour in *Eisenia fetida, Aporrectodea caliginosa* and *Lumbricus terrestris* when exposed to imidacloprid. *J. Soils Sediments* 12, 198–206. doi: 10.1007/s11368-011-0437-1

Dittbrenner, N., Schmitt, H., Capowiez, Y., and Triebskorn, R. (2011). Sensitivity of *Eisenia fetida* in comparison to *Aporrectodea caliginosa* and *Lumbricus terrestris* after imidacloprid exposure. body mass change histopathol. *J. Soils Sediments* 11, 1000–1010. doi: 10.1007/s11368-011-0397-5

Dittbrenner, N., Triebskorn, R., Moser, I., and Capowiez, Y. (2010). Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*). *Ecotoxicology* 19, 1567–1573. doi: 10.1007/s10646-010-0542-8

Doan, T. T., Ngo, P. T., Rumpel, C., Van Nguyen, B. T., and Jouquet, P. (2013). Interactions between compost, vermicompost and earthworms influence plant growth and yield: a on-year greenhouse experiment. *Sci. Hortic.* 160, 148–154. doi: 10.1016/j.scienta.2013.05.042

Druille, M., Omacini, M., Golluscio, R. A., and Cabello, M. N. (2013). Arbuscular mycorrhizal fungi are directly and indirectly affected by glyphosate application. *Appl. Soil Ecol.* 72, 143–149. doi: 10.1016/j.apsoil.2013.06.011

Easton, A. H., and Goulson, D. (2013). The neonicotinoid insecticide imidacloprid repels pollinating flies and beetles at field-realistic concentrations. *PLoS ONE* 8:e54819. doi: 10.1371/journal.pone.0054819

Edwards, C. A., and Fletcher, K. E. (1988). Interactions between earthworm and microorganisms in organic-matter breakdown. *Agric. Ecosyst. Environ.* 24, 235–247. doi: 10.1016/0167-8809(88)90069-2

Eijsackers, H., Beneke, P., Maboeta, M., Louw, J. P., and Reinecke, A. J. (2005). The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality. *Ecotoxicol. Environ. Saf.* 62, 99–111. doi: 10.1016/j.ecoenv.2005.02.017

Eisenhauer, N., Marhan, S., and Scheu, S. (2008). Assessment of anecic behavior in selected earthworm species: effects on wheat seed burial, seedling establishment, wheat growth and litter incorporation. *Appl. Soil Ecol.* 38, 79–82. doi: 10.1016/j.apsoil.2007.07.002

Elbert, A., Haas, M., Springer, B., Thielert, W., and Nauen, R. (2008). Applied aspects of neonicotinoid uses in crop protection. *Pest Manag. Sci.* 64, 1099–1105. doi: 10.1002/ps.1616

Forey, E., Barot, S., Decaëns, T., Langlois, E., Laossi, K.-R., Margerie, P., et al. (2011). Importance of earthworm–seed interactions for the composition and structure of plant communities: a review. *Acta Oecologica* 37, 594–603. doi: 10.1016/j.actao.2011.03.001

Furlan, L., and Kreutzweiser, D. (2015). Alternatives to neonicotinoid insecticides for pest control: case studies in agriculture and forestry. *Environ. Sci. Pollut. Res.* 22, 135–147. doi: 10.1007/s11356-014-3628-7

Gao, M. L., Song, W. H., Zhang, J. Y., and Guo, J. (2013). Effect on enzymes and histopathology in earthworm (*Eisenia foetida*) induced by triazole fungicides. *Environ. Toxicol. Pharmacol.* 35, 427–433. doi: 10.1016/j.etap.2013.02.003

Gaupp-Berghausen, M., Hofer, M., Rewald, B., and Zaller, J. G. (2015). Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. *Sci. Rep.* 5:12886. doi: 10.1038/srep12886

Gill, R. J., Ramos-Rodriguez, O., and Raine, N. E. (2012). Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature* 491, 105–108. doi: 10.1038/nature11585

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol.* 50, 977–987. doi: 10.1111/1365-2664.12111

Green, J. A., Minear, K., and Okazaki, N. (2008). Effects of herbicide and pesticide on the common earthworm, *Lumbricus* sp. *Ergo.* 3, 70–77.

Griffiths, B. S., Bonkowski, M., Roy, J., and Ritz, K. (2001). Functional stability, substrate utilisation and biological indicators of soils following environmental impacts. *Appl. Soil Ecol.* 16, 49–61. doi: 10.1016/S0929-1393(00)00081-0

Hättenschwiler, S., Tiunov, A. V., and Scheu, S. (2005). Biodiversity and litter decomposition in terrestrial ecosystems. *Annu. Rev. Ecol. Evol. Systemat.* 36, 191–218. doi: 10.1146/annurev.ecolsys.36.112904.151932

Imparato, V., Santos, S. S., Johansen, A., Geisen, S., and Winding, A. (2016). Stimulation of bacteria and protists in rhizosphere of glyphosate-treated barley. *Appl. Soil Ecol.* 98, 47–55. doi: 10.1016/j.apsoil.2015.09.007

Jänsch, S., Frampton, G. K., Römbke, J., Van Den Brink, P. J., and Scott-Fordsmand, J. J. (2006). Effects of pesticides on soil invertebrates in model ecosystem and field studies: a review and comparison with laboratory toxicity data. *Environ. Toxicol. Chem.* 25, 2490–2501. doi: 10.1897/05-439R.1

Keuskamp, J. A., Dingemans, B. J. J., Lehtinen, T., Sarneel, J. M., and Hefting, M. M. (2013). Tea Bag Index: a novel approach to collect uniform decomposition data across ecosystems. *Methods Ecol. Evol.* 4, 1070–1075. doi: 10.1111/2041-210X.12097

Köhler, H. R., and Triebskorn, R. (2013). Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? *Science* 341, 759–765. doi: 10.1126/science.1237591

Krupinsky, J. M., Bailey, K. L., Mcmullen, M. P., Gossen, B. D., and Turkington, T. K. (2002). Managing plant disease risk in diversified cropping systems. *Agron. J.* 94, 198–209. doi: 10.2134/agronj2002.0198

Laossi, K. R., Ginot, A., Noguera, D. C., Blouin, M., and Barot, S. (2010). Earthworm effects on plant growth do not necessarily decrease with soil fertility. *Plant Soil* 328, 109–118. doi: 10.1007/s11104-009-0086-y

Liu, Z., Dai, Y., Huang, G., Gu, Y., Ni, J., Wei, H., et al. (2011). Soil microbial degradation of neonicotinoid insecticides imidacloprid, acetamiprid, thiacloprid and imidaclothiz and its effect on the persistence of bioefficacy against horsebean aphid *Aphis craccivora* Koch after soil application. *Pest Manag. Sci.* 67, 1245–1252. doi: 10.1002/ps.2174

Merrington, G., Rogers, S. L., and Van Zwieten, L. (2002). The potential impact of long-term copper fungicide usage on soil microbial biomass and microbial activity in an avocado orchard. *Aust. J. Soil Res.* 40, 749–759. doi: 10.1071/SR01084

Milcu, A., Schumacher, J., and Scheu, S. (2006). Earthworms (Lumbricus terrestris) affect plant seedling recruitment and microhabitat heterogeneity. *Funct. Ecol.* 20, 261–268. doi: 10.1111/j.1365-2435.2006.01098.x

Moore, L. J., Fuentes, L., Rodgers, J. H. J., Bowerman, W. W., Yarrow, G. K., Chao, W. Y., et al. (2012). Relative toxicity of the components of the original formulation of Roundup to five North American anurans. *Ecotoxicol. Environ. Saf.* 78, 128–133. doi: 10.1016/j.ecoenv.2011.11.025

Morowati, M. (2000). Histochemical and histopathological study of the intestine of the earthworm (*Pheretima elongata*) exposed to a field dose of the herbicide glyphosate. *Environmentalist* 20, 105–111. doi: 10.1023/A:1006704009184

Mullin, C. A., Fine, J. D., Reynolds, R. D., and Frazier, M. T. (2016). Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. *Front. Public Health* 4:92. doi: 10.3389/fpubh.2016.00092

Paul, E.A. (2015). *Soil Microbiology, Ecology, and Biochemistry*. London: Academic Press.

Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., and Vandenbulcke, F. (2014). Pesticides and earthworms. a review. *Agron. Sustain. Dev.* 34, 199–228. doi: 10.1007/s13593-013-0151-z

Pelosi, C., Toutous, L., Chiron, F., Dubs, F., Hedde, M., Muratet, A., et al. (2013). Reduction of pesticide use can increase earthworm populations in wheat crops in a European temperate region. *Agric. Ecosyst. Environ.* 181, 223–230. doi: 10.1016/j.agee.2013.10.003

Piola, L., Fuchs, J., Oneto, M. L., Basack, S., Kesten, E., and Casabé, N. (2013). Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. *Chemosphere* 91, 545–551. doi: 10.1016/j.chemosphere.2012.12.036

Pisa, L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Downs, C. A., Goulson, D., et al. (2015). Effects of neonicotinoids and fipronil on non-target invertebrates. *Environ. Sci. Pollut. Res.* 22, 68–102. doi: 10.1007/s11356-014-3471-x

Prescott, C. E. (2010). Litter decomposition: what controls it and how can we alter it to sequester more carbon in forest soils? *Biogeochemistry* 101, 133–149. doi: 10.1007/s10533-010-9439-0

Sannino, F., and Gianfreda, L. (2001). Pesticide influence on soil enzymatic activities. *Chemosphere* 45, 417–425. doi: 10.1016/S0045-6535(01)00045-5

Santos, M. J., Morgado, R., Ferreira, N. G., Soares, A. M., and Loureiro, S. (2011). Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem. *Ecotoxicol. Environ. Saf.* 74, 1994–2001. doi: 10.1016/j.ecoenv.2011.06.003

Scheu, S. (1992). Automated measurement of the respiratory response of soil microcompartments: active microbial biomass in earthworm faeces. *Soil Biol. Biochem.* 24, 1113–1118. doi: 10.1016/0038-0717(92)90061-2

Scheu, S. (2003). Effects of earthworms on plant growth: patterns and perspectives. *Pedobiologia* 47, 846–856. doi: 10.1078/0031-4056-00270

Scheu, S., and Setälä, H. (2002). "Multitrophic interactions in decomposer food-webs," in *Multitrophic Level Interactions,* eds T. Tscharntke and B. Hawkins. (Cambridge, UK: C*ambridge University Press),* 223–264. doi: 10.1017/CBO9780511542190.010

Tu, C., Wang, Y., Duan, W. X., Hertl, P., Tradway, L., Brandenburg, R., et al. (2011). Effects of fungicides and insecticides on feeding behavior and community dynamics of earthworms: implications for casting control in turfgrass systems. *Appl. Soil Ecol.* 47, 31–36. doi: 10.1016/j.apsoil.2010.11.002

Van Der Sluijs, J. P., Amaral-Rogers, V., Belzunces, L. P., Lexmond, M. F. I. J. B. V., Bonmatin, J.-M., Chagnon, M., et al. (2015). Conclusions of the worldwide integrated assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. *Environ. Sci. Pollut. Res.* 22, 148–154. doi: 10.1007/s11356-014-3229-5

Van Groenigen, J. W., Lubbers, I. M., Vos, H. M. J., Brown, G. G., De Deyn, G. B., and Van Groenigen, K. J. (2014). Earthworms increase plant production: a meta-analysis. *Sci. Rep.* 4:6365. doi: 10.1038/srep06365

Wang, Y. H., Cang, T., Zhao, X. P., Yu, R. X., Chen, L. P., Wu, C. X., et al. (2012a). Comparative acute toxicity of twenty-four insecticides to earthworm, *Eisenia fetida*. *Ecotoxicol. Environ. Saf.* 79, 122–128. doi: 10.1016/j.ecoenv.2011.12.016

Wang, Y. H., Wu, S. G., Chen, L. P., Wu, C. X., Yu, R. X., Wang, Q., et al. (2012b). Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. *Chemosphere* 88, 484–491. doi: 10.1016/j.chemosphere.2012.02.086

Whitehorn, P. R., O'Connor, S., Wäckers, F. L., and Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* 336, 351–352. doi: 10.1126/science.1215025

Yasmin, S., and D'souza, D. (2007). Effect of pesticides on the reproductive output of *Eisenia fetida*. *Bull. Environ. Contam. Toxicol.* 79, 529–532. doi: 10.1007/s00128-007-9269-5

Zabaloy, M. C., Gomez, E., Garland, J. L., and Gomez, M. A. (2012). Assessment of microbial community function and structure in soil microcosms exposed to glyphosate. *Appl. Soil Ecol.* 61, 333–339. doi: 10.1016/j.apsoil.2011.12.004

Zaller, J. G., and Arnone, J. A. (1999). Earthworm and soil moisture effects on the productivity and structure of grassland communities. *Soil Biol. Biochem.* 31, 517–523. doi: 10.1016/S0038-0717(98)00126-6

Zaller, J. G., Heigl, F., Ruess, L., and Grabmaier, A. (2014). Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Sci. Rep.* 4:5634. doi: 10.1038/srep05634

Zaller, J. G., König, N., Tiefenbacher, A., Muraoka, Y., Querner, P., Ratzenböck, A., et al. (2016). Pesticide seed dressings can affect the activity of various soil organisms and reduce decomposition of plant material. *BMC Ecol.* 16:37, doi: 10.1186/s12898-12016-10092-x

Zaller, J. G., Parth, M., Szunyogh, I., Semmelrock, I., Sochurek, S., Pinheiro, M., et al. (2013). Herbivory of an invasive slug is affected by earthworms and the composition of plant communities. *BMC Ecol.* 13, 20. doi: 10.1186/1472-6785-13-20

Zaller, J. G., and Saxler, N. (2007). Selective vertical seed transport by earthworms: implications for the diversity of grassland ecosystems. *Eur. J. Soil Biol.* 43, S86–S91. doi: 10.1016/j.ejsobi.2007.08.010

Zang, Y., Zhong, Y., Luo, Y., and Kong, Z. M. (2000). Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. *Environ. Pollut.* 108, 271–278. doi: 10.1016/S0269-7491(99)00191-8

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AT declared a shared affiliation, though no other collaboration, with one of the authors JL to the handling Editor, who ensured that the process nevertheless met the standards of a fair and objective review.

Copyright © 2017 Van Hoesel, Tiefenbacher, König, Dorn, Hagenguth, Prah, Widhalm, Wiklicky, Koller, Bonkowski, Lagerlöf, Ratzenböck and Zaller. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 18 October 2017
doi: 10.3389/fenvs.2017.00062

Nocturnal Risks-High Bat Activity in the Agricultural Landscape Indicates Potential Pesticide Exposure

*Peter Stahlschmidt, Melanie Hahn and Carsten A. Brühl**

Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany

Although agriculture dominates much of Europe's landscape, there is virtually no information on foraging activity of bats in different crops. Additionally little is known about pesticide exposure of bats and related effects and there are currently no specific regulatory requirements to include bats in European Union pesticide risk assessments for the registration of these chemicals although other mammals are considered. To evaluate the potential pesticide exposure of bats, we studied bat diversity and activity as well as the availability of aerial prey insects in different crops and semi-natural habitats in south-western Germany in a landscape dominated by agriculture. In 300 accumulated sampling nights more than 24,000 bat call sequences were acoustically recorded and, in parallel, almost 110,000 insects of suitable prey sizes were sampled by light traps. A total of 14 bat species were recorded, among them the locally rare and for Germany critically endangered northern bat (*Eptesicus nilssonii*) and the barbastelle (*Barbastella barbastellum*), all of them also occurring over agricultural fields. In comparison to agricultural habitats, higher activity levels in forest sites were only found for *Myotis* species but not for species of the genera *Pipistrellus, Eptesicus* and *Nyctalus*. There were no significant differences in the availability of aerial nocturnal insects between forest, meadow and agricultural habitats. Comparing the different agricultural crops, significantly fewer bat call sequences and lower numbers of nocturnal insects were collected above the vineyards compared to orchards, cereal and vegetable fields. Highest activity levels of all bat species were recorded above agricultural fields situated next to forests. Given the high bat activity levels recorded at several agricultural sites, among them orchard and vegetable fields both known for their high pesticide inputs, and the availability of suitable prey insects, we conclude that pesticide exposure via ingestion of contaminated insect prey is possible. This potential risk is currently not considered in the European pesticide risk assessment scheme.

Keywords: chiroptera, crops, pesticide, risk assessment

OPEN ACCESS

Edited by:
Veerle L.B. Jaspers, Norwegian University of Science and Technology, Norway

Reviewed by:
Markus Wagner, NERC Centre for Ecology & Hydrology, United Kingdom
Richard Shore, CEH, United Kingdom
Davi Castro Tavares, State University of Norte Fluminense, Brazil

****Correspondence:***
Carsten A. Brühl
bruehl@uni-landau.de

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *12 August 2016*
Accepted: *22 September 2017*
Published: *18 October 2017*

Citation:
Stahlschmidt P, Hahn M and Brühl CA (2017) Nocturnal Risks-High Bat Activity in the Agricultural Landscape Indicates Potential Pesticide Exposure. Front. Environ. Sci. 5:62. doi: 10.3389/fenvs.2017.00062

INTRODUCTION

Carson's (1962) classic book Silent Spring has immortalized the detrimental effects of organochlorine pesticides on the environment in general and on birds in particular. In the 1960s and 1970s it was also demonstrated that these pesticides were responsible for significant mortality of some bat populations in Europe and the USA (e.g., Jefferies, 1972; Gelusco et al., 1976; Clark et al., 1978). The detrimental highly toxic and persistent pesticides have been replaced by modern

pesticides in the European Union and many other countries in the 1970s and 1980s. In recent decades, however, applications of pesticides have even increased even more and, simultaneously, the agricultural landscape heterogeneity has been greatly reduced (Benton et al., 2003). Both aspects of agricultural intensification have been associated with further declines in biodiversity and are sometimes referred to as the Second Silent Spring (e.g., for farmland birds; Krebs et al., 1999). So far, little is known about the relative contribution of habitat loss and use of chemicals to the negative effects on biodiversity. Recently, Geiger et al. (2010) examined the impacts of several factors of agricultural intensification on EU level and identified the use of pesticides as the most consistent to have negative effects on species diversity.

The need for assessing the risk of pesticide exposure on non-target organisms is recognized by regulatory agencies such as the European Food and Safety Authority (EFSA). No authorisation for new pesticides is granted unless a risk assessment demonstrates that no risk for wildlife species occurs when the pesticide is applied under field conditions (European Food Safety Authority, 2009). The current procedure also includes a risk assessment for birds and mammals where, insectivorous mammals are represented by shrews (European Food Safety Authority, 2009). No reference at all is made to bats, although they are reported as being threatened by pesticides (e.g., O'Shea and Johnson, 2009) and comprise one-fifth off all European mammal species with a very specific ecology including hibernation and a low reproduction rate (only a single offspring per year). The reason for this omission is probably related to the scarcity of ecological data and limited knowledge about the occurrence and activity of bats in agricultural crops. However an ecological vulnerability analysis for wildlife using autecological information revealed that bats were among the most vulnerable taxa for studied pesticides (DeLange et al., 2009). Therefore information about the presence of an organism group in or near crops is crucial for the assessment of potential exposure and effects of pesticides.

To estimate the pesticide exposure of bats through ingestion of potentially contaminated insects (oral exposure) we therefore first need to know which species occur in which crop and to what extent. Hence, in this study we recorded bat activity and additionally the availability of nocturnal prey insects in a multitude of agricultural sites and compared them to simultaneous recordings (same sampling night) in nearby habitats know to be used for foraging such as forests and meadows. Furthermore, we examined if recorded bat activity in the agricultural landscape is related to habitat type (i.a. forest, forest edge and open landscape), crop, and nocturnal insect abundance.

MATERIALS AND METHODS

Study Sites and Sampling

The study was conducted in an agricultural landscape in Rhineland-Palatinate, SW Germany around Landau (Pfalz; 49°11.9064′ N, 8°7.0152′ E). The climate of the region is characterized by an average annual temperature of 10°C and a precipitation of 700 mm. The sampling sites were distributed in 6 sampling areas, being at least 6 km apart from each other. Each sampling area comprised 10 sampling sites, 8 in agricultural fields and one sampling area situated in a forest and another one situated in a meadow (referred to as semi-natural habitats). These were used to compare the recorded activity levels of the examined agricultural fields to activity levels of habitats know to be used for foraging. To allow direct comparison of bat activity in the different habitats, all sites in an area were sampled simultaneously during one night. In order to consider temporal variability each area was surveyed 5 times, resulting in a total of 300 sampling nights (6 areas × 10 sites × 5 nights). All sites were located less than 2.5 km away from the closest village and the closest forest of each area, assuring they were within the home range of all native bat species having their roost sites in settlements or forests. The distance of 2.5 km is based on the foraging range of the common pipistrelle (*Pipistrellus pipistrellus*), the species with the shortest maximum distance (2.5 km) between foraging sites and roost sites among the native species (Racey and Swift, 1985; Dietz et al., 2007). Agricultural sampling sites (apple orchards, vineyards, cereal-, and vegetable fields) were chosen to reflect the coverage of the different crops in each sampling area. The forest sites were mixed deciduous forest of different age and stand structure with European beech *(Fagus sylvatica)* being the dominant tree species. The meadow sites were agricultural grasslands with differing management intensities. After analyzing the data we realized that proximity to forest has a high influence on bat activity. Two of the cereal sampling sites were situated 100 m away from a forest. Data of these sites were therefore separately analyzed and termed "forest edge."

At each site, bat activity and nocturnal insect availability was assessed simultaneously, with the insect traps being at least 50 m away from the batcorders to avoid increased and biased bat activity pattern through attraction of the trap light. Batcorders and light traps were situated at least 40 m from the field edge. The recordings of bat activity and the sampling of nocturnal insects were performed from sunset to sunrise. In a few cases ($n = 3$) light traps did not work the whole night so that individual samples had to be rejected from the analysis. The study was conducted from the beginning of June until the end of August 2008, coinciding with the lactation period for most European bats (Vaughan et al., 1997). All sampling and recording was conducted in nights with temperatures above 16°C at sunset, no rain and a low wind speed (below 10 km/h).

Bat Activity Measurement

Acoustic measurement of bat activity is a reliable estimate of foraging activity (Russo and Jones, 2003). Bat activity was recorded by using 10 automatic stationary bat detector systems, so-called batcorders (ecoObs GmbH, Nürnberg, Germany) a method suitable to address spatial and temporal variation in bat activity pattern (Stahlschmidt and Brühl, 2012a). Batcorders were installed at a height of 3.5 m above ground and adjusted to the system's standard settings (Runkel, 2008). The sampling points were chosen in a way that assured uncluttered acoustic space within the detection radius of the system, i.e., 10 m (Runkel, 2008). The activity was measured as the number of recorded call-sequences per night. The software bcDiscriminator

(ecoObs GmbH, Nürnberg, Germany) was used to automatically determine bat species by their specific calls and to exclude the non-bat recordings. All doubtful determinations were manually identified by using the software bcAnalyze and by comparing sonograms and oscillograms of the calls with images from Skiba (2009). For statistical analyses the individual bat calls were assigned to the following species groups since it was not possible to identify all calls with sufficient probability to species level: *Pipistrellus, Eptesicus-Nyctalus* and *Myotis*. Species of the first two groups are predominately aerial hawker while the recorded *Myotis* species are more adapted to high-clutter environments such as forests. The group *Eptesicus-Nyctalus* included two genera that have similar food preferences and are also acoustically very similar and cannot be discriminated always with certainty.

Insect Sampling

Simultaneously to the bat recording, we measured the availability of nocturnal aerial insects using unattended light traps. Each light trap consisted of two ultraviolet fluorescent tubes (12V, 15W), two crossed acryl glasses and a plastic bowl hanging below the light and filled with 2 L of water. Three drops of an odorless detergent was added to reduce surface tension and therefore minimize the escaping of caught insects (Hahn et al., 2017). Light traps were positioned at least 50 m within the crop field and installed at a height of 1.8 m. The used light traps have an attraction radius below 15 m as evaluated in previous experiments. To assure that only nocturnal insects were sampled, the traps were automatically activated at dusk and deactivated at dawn. Insects other than Diptera or macro-moths were identified to order, Diptera to sub-order and macro-moths to family level. Furthermore, insect size was measured individually and insects were assigned to defined size classes.

The prey size suitable for *Pipistrellus*-group is reported to be around 3 mm on average (Barlow, 1997) and mainly <5 mm (Beck, 1995). Thus, the main prey size was considered to be 2–5 mm. The species of the *Eptesicus-Nyctalus* group differ in their preferred prey, but all of them include small Diptera (the most frequently recorded insect group in our study) in their diet and generally seem to consume different insects in the proportions encountered (Dietz et al., 2007 and references therein). Therefore, insects larger than 2 mm of all orders were considered as potential prey for *Eptesicus-Nyctalus*. Not all recorded *Myotis* species are aerial hunters and their prey could not be assessed by the applied insect trapping method. Since it was not possible to identify all *Myotis* calls with sufficient probability to species level and, consequently to assign them to groups with similar prey preferences, they were excluded from this analysis of food availability and bat activity.

Statistical Analysis

Permutational multivariate analysis of variance (PERMANOVA Anderson, 2001) was used to assess differences in (1) activities of the bat groups (*Pipistrellus; Eptesicus-Nyctalus, Myotis*) between the different habitat types (forest, forest edge, open landscape), (2) activities of the bat groups between the examined open landscape habitats (meadow, vineyard, cereal fields, vegetable fields, orchards), and (3) the differences in nocturnal insect availability (insects of the size class 2–5 mm, all insects) between the habitats (forest, forest edge, meadow, vineyard, cereal fields, vegetable fields, orchards). PERMANOVA is a non-parametric method that can be used for univariate and multivariate questions. PERMANOVA is a routine for testing the simultaneous response of one or more variables to one or more factors in an analysis of variance (ANOVA) experimental design on the basis of any resemblance measure, using permutation methods. Analysis of variance with permutations (PerANOVA) was used since the data were not normally distributed.

The Euclidean dissimilarity measure was used as the distance metric with 999 permutations for the probability tests for the univariate analysis. The factors (habitat types, open landscape habitats, insect availability) were treated as fixed, the sampling replication were nested within sites. When a factor was identified as significant (at $\alpha = 0.05$), post-hoc pairwise tests (t-test) were conducted, again using 999 permutations. Analyses were conducted using the software packages PRIMER 6 (version 6.1.13) and PERMANOVA+ (version 1.0.3).

Spearman's coefficient correlation was used to explore relationship between site specific and log transformed mean bat activities of *Pipistrellus* and *Eptesicus-Nyctalus* and availabilities of nocturnal insects of the size class 2–5 mm and total number of insects, respectively. These analyses were conducted using SPSS ver. 17 (SPSS, Chicago, USA).

RESULTS

Bat Activity

In 300 sampling nights a total of 24,012 call sequences were recorded, corresponding to 14 species (**Table 1**). About 66.6% of them were assigned to *Pipistrellus*, 26.3% to *Eptesicus-Nyctalus*, 6.1% to *Myotis*, and 0.3% to *Plecotus*. *Barbabastella barbastellus* was only recorded 3 times. The remaining 0.6% sequences were unidentifiable and thus excluded from the analysis. By far the most detected species was *Pipistrellus pipistrellus* with 65.0% of all recorded call sequences.

Apart from the common pipistrelle, Nathusius's bat (*Pipistrellus nathusii*) and the midge bat (*Pipistrellus pygmaeus*) of the genus *Pipistrellus* were detected (**Table 1**). On average, the highest numbers of total *Pipistrellus* call sequences were recorded at forest edges, the lowest numbers above vineyards (**Table 1**). Relatively high numbers were detected in the orchards while forests, meadows, cereal and vegetable fields were used to similarly extents (**Table 1**).

In the species group *Eptesicus-Nyctalus* we recorded the serotine (*Eptesicus serotinus*), the northern bat (*Eptesicus nilssonii*), the noctule (*Nyctalus noctula*), and Leisler's bat (*Nyctalus leisleri*). The highest median numbers of call sequences of all *Eptesicus-Nyctalus* were recorded at the forest edges. For all species of that group similar activities were detected in forests and open landscape habitats (**Table 1**).

The differences in activity levels between habitat types (forest, forest edge, open landscape) were significant for the groups *Pipistrellus* and *Eptesicus-Nyctalus* (PERMANOVA: $P > 0.005$ in both cases). Pair-wise comparisons (PERMANOVA) showed no differences between open landscape and forest ($P = 0.883$ and

TABLE 1 | Total number, median, and lower and upper quartile of bat call sequences per habitat of 5 sampling nights per site for all sites (forest, $n = 6$; forest edge, $n = 2$; meadow, $n = 6$; vineyard, $n = 13$; orchard, $n = 5$; vegetable, $n = 19$; cereal, $n = 9$).

		Pipistrellus pipistrellus	*Pipistrellus nathusii*	*Pipistrellus pygmaeus*	*Pipistrellus spp.*	*Pipistrellus (total)*	*Eptesicus serotinus*	*Eptesicus nilssonii*	*Nyctalus noctula*	*Nyctalus leisleri*	*Eptesicus /Nyctalus spp.*	*Eptesicus /Nyctalus (total)*	*Myotis mystacinus /brandtii*	*Myotis daubentonii*	*Myotis bechsteinii*	*Myotis nattereri*	*Myotis myotis*	*Myotis spp.*	*Myotis (total)*	*Plecotus austriacus*	*Barbastella barbastellus*
$n = 6$	Total	1108	7	1	18	1134	199	54	121	25	349	748	147	95	112	93	8	176	631	2	1
	Lower quart	7,25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Forest	Median	18	0	0	0	0	2,5	0	0	0	2,5	0	1,5	1	0	1	0	2,5	1	0	0
	Upper quart	73	0	0	0	2	6	0	1,75	0	11,3	3	4	3	1,75	2,75	0	6	3	0	0
$n = 2$	Total	5716	47	8	18	5789	400	19	214	79	290	1002	37	34	30	23	3	59	186	2	2
	Lower quart	253	0	0	0,25	0	14	0	1	0	8,5	0	0	0	0	0	0	4,25	0	0	0
Edge	Median	540	1,5	0	1,5	2	26,5	0	8,5	0,5	18,5	7,5	2,5	1,5	0	1	0	6	1	0	0
	Upper quart	777	7,5	0	2	29,8	32	3,75	22	7,25	23,8	22,3	6,5	4,25	0	4,25	0,75	8,5	5,25	0	0
$n = 6$	Total	602	16	2	7	627	371	75	91	19	424	980	15	19	7	10	2	20	73	2	2
	Lower quart	2	0	0	0	0	2,25	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Meadow	Median	8,5	0	0	0	0	8,5	0	0	0	7	1	0	0	0	0	0	0	0	0	0
	Upper quart	19,5	0	0	0	1	15,5	0,75	3,5	0	19,5	7	0,75	0,75	0	0	0	1	0	0	0
$n = 13$	Total	615	17	0	3	635	137	4	31	17	174	363	18	2	7	26	5	19	77	47	0
	Lower quart	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Vineyard	Median	6	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
	Upper quart	12	0	0	0	1	2	0	0	0	3	1	0	0	0	0	0	0	0	0	0
$n = 5$	Total	3733	51	14	37	3835	212	10	60	18	203	503	33	12	0	31	0	33	109	2	0
	Lower quart	15,5	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Orchard	Median	29	0	0	0	0	3	0	0	0	4,5	0	0	0	0	0	0	0	0	0	0
	Upper quart	72,3	2	0	0	8	10,3	0	0	0	8	4	1	0	0	0	0	1,75	0	0	0
$n = 19$	Total	2812	88	13	21	2934	666	53	400	32	800	1951	95	30	5	28	55	119	332	8	0
	Lower quart	8	0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Vegetable	Median	16	0	0	0	0	4	0	0	0	6	0	0	0	0	0	0	0	0	0	0
	Upper quart	41	1	0	0	4	8	0	5,5	0	11	5	1	0	0	0	0	1	0	0	0
$n = 9$	Total	1263	39	4	4	1310	341	12	227	38	341	959	24	15	0	4	5	28	76	3	0
	Lower quart	7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Cereal	Median	12	0	0	0	0	2	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	Upper quart	32	1	0	0	3	12	0	5	0	11	4	0	0	0	0	0	0	0	0	0

$P = 0.401$, respectively), between forest edge and forest ($P = 0.036$ and $P = 0.062$, respectively) but between forest edge and open landscape ($P = 0.005$ and $P = 0.003$, respectively), caused by the high number of recorded call sequences for both groups at the forest edge habitats (**Table 1**).

Significant differences in activity patterns between the different habitats of the open landscape (crops and meadows) were also found for the groups *Pipistrellus* and *Eptesicus-Nyctalus* (PERMANOVA: $P = 0.011$ and $P = 0.005$, respectively). Pair-wise comparisons revealed that the vineyards differ in number of *Pipistrellus* call sequences from all other open landscape habitats (**Table 2**), revealing lowest activity levels. The same pattern was found for *Eptesicus-Nyctalus* with the exception that there was no difference in activity between the vineyards and orchards (**Table 2**).

The genus *Myotis* was represented by the whiskered bat (*Myotis mystacinus and brandtii*), Daubenton's bat (*Myotis daubentonii*), Bechstein's bat (*Myotis bechsteinii*), Natterer's bat (*Myotis nattereri*) and the greater mouse-eared bat (*Myotis myotis*). All *Myotis* species showed high activity for forest and forest edge habitats with the exception of the greater mouse-eared bat with slightly higher activity over vegetable fields (**Table 1**). Bechstein's bat was almost exclusively recorded in forests and at forest edges (**Table 1**). Mean number of call sequences of the gray long-eared bat (*Plecotus austriacus*) was highest over vineyards (**Table 1**). The barbastelle (*B. barbastellus*) was only recorded twice at forest edges and once in a forest (**Table 1**).

Activity levels between habitat types were different for *Myotis* (PERMANOVA: $P = 0.001$). Pair-wise comparison (PERMANOVA) demonstrated no differences between forest edge and forest ($P = 0.918$) but between open landscape and forest ($P = 0.001$) and between open landscape and forest edge ($P = 0.003$) which could be attributed to the low activity levels recorded at the open landscape. No differences were found between *Myotis* call sequences at the different open landscape habitats (PERMANOVA: $P = 0.162$), which were lower compared to those in the forests and at the forest edges (**Table 1**).

When comparing the summed bat activity pattern for the five nights of all examined habitats which were simultaneously recorded in each sampling area, the highest activity levels were recorded at forest edges (sampling areas 1 and 2), over vegetable fields (sampling areas 3 and 4), an orchard (sampling area 5) and within a forest (sampling area 6).

Food Availability

In total 109,264 insects with body size larger than 2 mm were trapped in 281 sampling nights (70,735 of them were assigned to the size class 2–5 mm). More than 70% of the sampled insects were assigned to the order Diptera. On average, the highest numbers of insects larger than 2 mm were recorded in forest habitats (**Table 3**). Numbers of insects of the size class 2–5 mm were highest in vegetable fields and forests (**Table 3**). For both size groups the lowest numbers of insects were collected in vineyards (**Table 3**). Availability of total nocturnal insects larger than 2 mm and insects of the size classes 2–5 mm, representing suitable prey for *Eptesicus-Nyctalus* and *Pipistrellus*, respectively, differed significantly between habitats (PERMANOVA: $P = 0.002$ and $P = 0.001$, respectively). Pair-wise comparisons revealed that this could be attributed to vineyards which differed from forest, meadow and other crops by lower insect abundances while no differences between the other three habitats were found (**Table 4**).

A significant positive correlation was found between site specific *Pipistrellus* activity and insect availability of the size class 2–5 mm ($rs = 0.340$, $p = 0.007$, $n = 60$; **Figure 1A**) and site specific *Eptesicus-Nyctalus* activity and all insects larger than 2 mm ($rs = 0.484$, $p = 0.001$, $n = 60$, **Figure 1B**).

DISCUSSION

Farmland is the most widespread terrestrial wildlife habitat in Europe, covering 43% of the EU member states' surface area (Geiger et al., 2010). For bats, however, little is known about the role of agricultural crop fields as foraging habitats (Park, 2015). In contrary, the use of freshwater habitats or deciduous forests, both generally representing only small portions of most European landscapes, are well studied (e.g., Stahlschmidt et al., 2012; Zehetmair et al., 2015). Some studies have reported an avoidance of intensively managed agricultural fields by bats (Walsh and Harris, 1996; Vaughan et al., 1997). However, results of Vaughan et al. (1997) showed that bat activity levels over

TABLE 2 | Results of pairwise comparisons (PERMANOVA) of activity levels of the 3 bat groups (*Pipistrellus*, *Eptesicus-Nyctalus*, *Myotis, see above*) between the different open landscape habitats (meadow, vineyard, orchard. vegetable, cereal).

	Pipistrellus		*Eptesicus-Nyctalus*		*Myotis*	
	P(perm)	*t*	P(perm)	*t*	P(perm)	*t*
Vineyard-meadow	**0.001**	5.341	**0.001**	3.593	0.179	1.490
Vineyard-vegetable	**0.001**	3.076	**0.002**	3.417	0.027	2.347
Vineyard-cereal	**0.001**	3.084	**0.002**	3.343	0.659	0.552
Vineyard-orchard	**0.002**	2.221	0.051	2.228	0.059	1.871
Orchard-cereal	0.077	1.501	0.259	1.136	0.425	0.927
Orchard-meadow	0.032	1.296	0.132	1.604	0.613	0.550
Orchard-vegetable	0.055	2.181	0.272	1.102	0.976	0.054
Cereal-meadow	0.481	0.721	0.324	1.050	0.689	0.477
Cereal-vegetable	0.898	0.157	0.908	0.127	0.199	1.372
Meadow-vegetable	0.451	0.836	0.158	1.430	0.464	0.762

Significant values in bold.

TABLE 3 | Mean numbers and standard deviations (in brackets) of nocturnal insects per habitat.

Size class	Forest	Meadow	Vineyard	Orchard	Vegetable	Cereal
>2 mm	644 (150)	390 (277)	161 (33)	386 (119)	496 (320)	372 (194)
2–5 mm	353 (102)	248 (152)	82 (15)	271 (76)	354 (226)	262 (150)

Mean numbers of insects per habitat were calculated as the mean of all sampling nights (n = 4-5 per site) and all sites per habitats (forest: n = 6; meadow: n = 6; vineyard: n = 14; orchard: n = 5; vegetable: n = 19; cereal: n = 10).

TABLE 4 | Results of pairwise comparisons (PERMANOVA) of numbers of nocturnal insects (insects larger than 2 mm; insects sized between 2 and 5 mm) between the different habitats (forest, meadow, vineyard, orchard, vegetable, cereal).

	Insects larger than 2 mm		Insects 2–5 mm	
	P(perm)	*t*	P(perm)	T
Vineyard-meadow	**0.001**	3.715	**0.001**	4.063
Vineyard-vegetable	**0.001**	4.370	**0.001**	4.435
Vineyard-cereal	**0.001**	4.129	**0.001**	4.154
Vineyard-orchard	**0.001**	6.119	**0.002**	6.638
Vineyard-forest	**0.001**	9.249	**0.001**	9.331
Orchard-cereal	0.655	0.474	0.574	0.605
Orchard-meadow	0.756	0.431	0.742	0.418
Orchard-vegetable	0.163	1.387	0.183	1.357
Orchard-forest	0.055	3.139	0.052	2.726
Cereal-meadow	0.984	0.022	0.847	0.179
Cereal-vegetable	0.190	1.412	0.274	1.178
Cereal-forest	0.051	2.337	0.178	1.437
Vegetable-forest	0.620	0.545	0.816	0.230
Meadow-vegetable	0.306	1.084	0.314	1.087
Meadow-forest	0.074	1.928	0.142	1.575

Significant values in bold.

arable land in Great Britain were statistically lower for most bat species compared to their activities over water surfaces (i.e., rivers and lakes) but were comparable to the examined non-arable terrestrial habitats (different kinds of grassland and woodland). Water habitats are rare within most European agricultural landscapes while in contrast arable land constituting more than 40% of the available habitat (Walsh and Harris, 1996). Therefore, the predominant arable land, even if disproportionately more scarcely used by bats, may play an important and currently underestimated role as a foraging habitat. Wickramasinghe et al. (2003) compared bat activity across conventional and organic agricultural land and recorded higher activity on organic farms. However, subsequently, it was demonstrated that these differences were only due to higher activity over water habitats of the farms but not over land habitats (Davy et al., 2007). In one study in United Kingdom even higher bat activity levels were demonstrated on conventional farms when compared to farms using less intensive agricultural practices (Fuentes-Montemayor et al., 2011). Relatively large numbers of foraging attempts were recorded in some arable fields (Russo and Jones, 2003). Kalda et al. (2014) demonstrated the importance of woody habitats such as linear tree lines or solitary trees for bats in the agricultural landscape of Estonia. A recent study in Germany showed that open-space specialists foraged more intensively above agricultural fields during the migration period, while edge-space specialists foraged also during the energy demanding period of lactation (Heim et al., 2016). Here open-space and edge-space foraging migratory bats were recorded more often on farmland and arable fields than narrow-space foraging and regionally moving species which are able to forage within dense vegetation.

However, none of the aforementioned studies provides details about the crops in order to allow any conclusion about potential exposure of bats to pesticides. The present study is the first detailed investigation of the diversity and activity of European bats in different agricultural crops using a standardized approach that allows comparison to non-cropped semi-natural habitats.

Bat Activity

All 14 bat species recorded in the different habitats of the six sampling areas were also detected over agricultural fields, among them the northern bat, a species reported locally as facing extinction and the rare barbastelle which was not yet recorded in this region of Rhineland Palatinate (König and Wissing, 2007). Activity at a sampling site does not necessarily reflect its quality as a foraging habitat since quality is also reflected by the number of bat individuals present which depends on roost site availability and the distance to them. Therefore comparisons of site-specific activity levels of different habitat types on a large spatial scale are problematic (Hayes, 2000). However automated bat recording and our study design with several sampling sites in different habitats grouped in a sampling area within the home-range to potential roost sites (both housing and forests) for all occurring species, allows the direct comparison of activity levels between the different habitats.

The activity levels of the recorded species of the genera *Pipistrellus*, *Eptesicus* and *Nyctalus*, all of them being predominately aerial hawker, did not significantly differ between agricultural sites, forests and meadow habitats. Higher activity levels over agricultural fields than those in the simultaneously examined meadows and forests could even be demonstrated in several cases (fruit orchards, vegetable fields).

The activity levels of both aerial hawker groups (*Pipistrellus* and *Eptesicus-Nyctalus*) were correlated with suitable prey insect availability indicating that they use the agricultural sites for foraging. In accordance to the significant lower insect abundances found at the vineyards activity levels of the aerial hawkers were also significantly lower over vineyards compared to all other crop types.

In contrast, higher activity levels in the forests and significantly reduced activity in the open landscape were found for the *Myotis* species. Most of the recorded *Myotis* species are known to take their prey mainly (Natterer's and Bechstein's bat) or at least partly (Whiskered and Brandt's bat) by gleaning from vegetation (Dietz et al., 2007 and references therein). Bats using this foraging strategy are more adapted to high-clutter environments such as forests (e.g., in regards to their echolocation), but not to open landscape habitats (Aldridge and Rautenbach, 1987). Exceptions are the greater mouse-eared bat which almost exclusively feeds on carabid beetles and Daubenton's bat, a species adapted to take prey from water surfaces (Dietz et al., 2007 and references therein).

All examined bat groups showed remarkably high activity levels over agricultural fields located next to forests. Forest edges in general are known to be used for foraging by bat species that avoid navigating through structurally complex habitats as well as those that avoid the open landscape (Walsh and Harris, 1996; Morris et al., 2010).

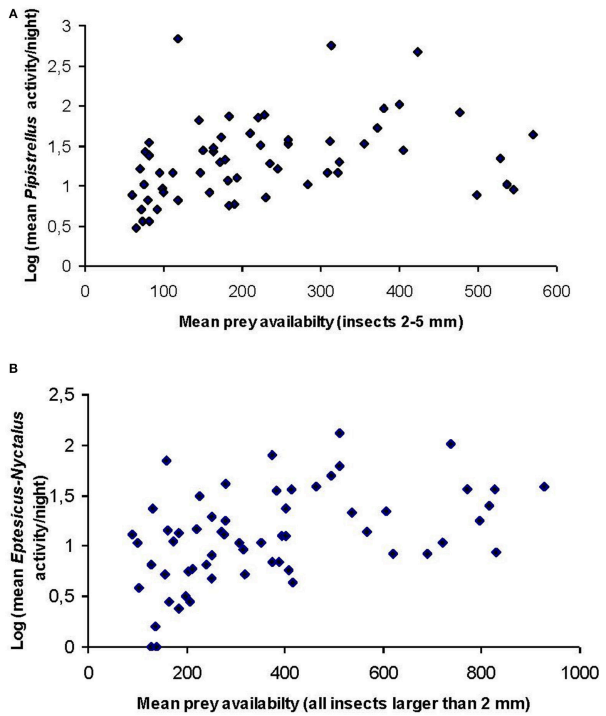


FIGURE 1 | Scatter plots of site-specific mean ($n = 5$ nights per site) bat activity of **(A)** *Pipistrellus* and **(B)** *Eptesicus-Nyctalus* against site-specific availability of the corresponding prey groups. Data were log transformed to account for the high variability at sites and sampling nights.

Food Availability

Abundances of insects of the examined size classes did not differ between forest, meadow and most agricultural habitats. This appears to be in contrast to other studies reporting insect abundances and diversity being negatively associated with agricultural intensification (e.g., Benton et al., 2002; Wickramasinghe et al., 2004). However, we compared abundance of nocturnal insects with more than 70% being Diptera. Diptera are collected in our light trap design (water filled bowl as collector) whereas this group might be underrepresented in other designs. In a study by Nielsen et al. (1994) the occurrence of Diptera was not significantly impacted by pesticide use and, while tillage has been reported as a disturbance factor for terrestrial Diptera, some species are even specialized on the initial stages of succession after tillage (Frouz, 1999). Thus some Diptera species may be less affected by agricultural intensification and occur in high abundances in crop fields. The main factors affecting the occurrence of Diptera with terrestrial larval stages are the organic matter content and the moisture of the soil (Frouz, 1999). The soils of vegetable fields are especially rich in organic matter due to the remnants of the former crops (up to 3 different vegetable cultures per year). In combination with the presence of permanently wet soils due to irrigation, vegetable fields appear to provide the most suitable conditions of the examined crops for Diptera leading in several cases to insect abundances even exceeding those measured simultaneously at nearby forest sites. The soils of the cereals fields are also relatively rich in organic matter due to the remnants of former crops while the orchards are poorer in this regard. Vineyards, however, do not provide suitable conditions for most Diptera since their soils are rather dry.

Potential Exposure to Pesticides

Given the high bat activity levels recorded at several agricultural sites and the availability of suitable prey insects, an uptake of pesticides through consumption of potentially contaminated food items after pesticide application is possible. Especially high bat activity levels were recorded in several apple orchards, a crop known for high pesticide input. Commercial apple plantations in Germany received for example applications of 30 pesticides (22 fungicides and 8 insecticides) in 2007 (Roßberg and Harzer, 2015). Because of the vegetation structure suitable for gleaning, orchards were the only crop where Natterer's and Brandt's bat were recorded on a regular basis. Since the estimation of the exposure requires information on pesticide residues on bat-specific food items, a follow-up study (Stahlschmidt and Brühl, 2012b) was performed in one of the apple orchards where high bat activity levels were demonstrated. According to the preferences of the recorded bat guilds the residue pattern of different nocturnal arthropod groups were examined following applications of insecticides. The highest residue values were measured on foliage-dwelling arthropods which may result in a risk for all bat species that, even to a small extent, include foliage-dwelling arthropods in their diet (Stahlschmidt and Brühl, 2012b). Chlorpyrifos, the insecticide used in this exposure study, was also evaluated in the first toxicity study performed with a bat species (Big brown bat, *Eptesicus fuscus*) and flight impairment was one of the endpoints (Eidels et al., 2016). The authors conclude that the field relevant applications of this insecticide "could present bats with dietary concentrations consistent with adverse effects."

Considering the high bat activity levels recorded over several vegetable fields indicating a good foraging habitat and the pesticide input in these crops (Roßberg, 2007), a study of pesticide residue patterns on nocturnal insects is strongly suggested to get a realistic estimate for the risk of pesticide exposure. The mean number of call sequences per night of the greater mouse-eared bat, a species almost exclusively feeding on carabid beetles (Beck, 1995), was highest above vegetable fields. Ground-dwelling arthropods such as carabid beetles may exhibit high pesticide residues especially after ground-directed applications in the afternoon. A massive die-off of juvenile greater mouse-eared bats which was attributed to the application of an organophosphate to potato fields and apple orchards in Germany (Hofmann, 1991) already demonstrated that this species is threatened by pesticide exposure. While in the orchards most of the airborne small insects were non-Diptera such as small moths (Hahn et al., 2017), Diptera were the predominant group in the vegetable fields. Since it has been shown that Diptera larvae can accumulate significant amounts of chemicals (Eitminavichiute et al., 1982; Park et al., 2009), food residue patterns in vegetable fields may differ from those measured in the

orchard. Research is required to examine if such an accumulation of modern and less persistent pesticides takes place in Diptera developing in agricultural soils, especially in vegetable fields where wet soils may increase the contact of the larvae with pesticides.

Bat activity was rather low over the vineyards with the exception of the gray long-eared bat. While availability of nocturnal insects in general was lower in vineyards compared to the other agricultural habitats, higher abundances of nocturnal moths of the family Noctuidae (Hahn et al., 2017), on which the gray long-eared bat is almost exclusively preying (Bauerová, 1982), were recorded. In the residue study performed in the apple orchard (Stahlschmidt and Brühl, 2012b) large moths exhibited the lowest pesticide residues of all examined arthropods groups, revealing the lowest risk for bat species mainly feeding on them. Therefore, similar low residue pattern on the moths and a low risk for the gray long-eared bat feeding on them are expected in vineyards.

Remarkably high activity levels of all examined bat groups were detected over agricultural fields located next to forests. Given that in agricultural landscapes most forest edges are situated next to crop fields, a thorough examination of the potential pesticide exposure is necessary and special risk mitigation methods for those habitats may be required. Forest edges function as windbreaks which potentially could concentrate large densities of contaminated insects after pesticide application. The northern bat and the barbastelle were in this study predominantly recorded at the forest edges. Both are rare species and a potential risk due to pesticide exposure could have severe impacts on their populations. Research is also required if Bechstein's bat and the brown long-eared bat (*Plecotus auritus*), both forest inhabiting bats exclusively taking their prey by gleaning, are using orchards situated next to forests for foraging since a high risk is expected due to the elevated residue values of foliage-dwelling arthropods in orchards (Stahlschmidt and Brühl, 2012b).

CONCLUSION

The present study demonstrated that abundances of suitable prey insects for aerial hunting bats in orchards, vegetable and cereal fields are comparable to nearby forests and meadows, the latter known to be used as foraging habitats by bats. Since high bat activity was recorded in orchards and arable fields, crops that are known for elevated pesticide inputs, an exposure through ingestion of pesticide contaminated insects is especially likely. The following scenarios indicate a risk of pesticide exposure for bats: gleaners foraging in orchards, bats preying on soil arthropods in vegetable fields, aerial hawkers feeding on Diptera over vegetable fields, and bat species foraging along forest edges situated next to agricultural fields. In addition to studies on the pesticide contamination of bat food items as a basis for the development of a realistic risk assessment approach for this group, telemetry studies are needed to gain insights in individual foraging patterns in agricultural habitats.

AUTHOR CONTRIBUTIONS

CB and PS designed the study. PS and MH collected the data. PS anaylsed the data. PS, MH, and CB wrote the manuscript.

FUNDING

We thank the Ministry of Science Rhineland-Palatinate for financial support in the project "Ecotoxicology in the agricultural landscape: from molecule to measures."

ACKNOWLEDGMENTS

The authors thank the farmers in the region for access to their land and local authorities for granting permission for road access. We are also thankful for the comments provided by five reviewers that helped to improve the manuscript.

REFERENCES

Aldridge, H. D. J. N., and Rautenbach, I. L. (1987). Morphology, echolocation and resource partitioning in insectivorous bats. *J. Anim. Ecol.* 56, 763–778. doi: 10.2307/4947

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Barlow, K. E. (1997). The diets of two phonic types of the bat *Pipistrellus pipistrellus* in Britain. *J. Zool.* 243, 597–609. doi: 10.1111/j.1469-7998.1997.tb02804.x

Bauerová, Z. (1982). Contribution to the trophic ecology of the grey long-eared bat, *Plecotus austriacus*. *Folia Zool. Brno*. 31, 113–122.

Beck, A. (1995). Fecal analysis of European bat species. *Myotis* 32–33, 109–119.

Benton, T. G., Bryant, D. M., Cole, L., and Crick, H. Q. P. (2002). Linking agricultural practice to insect and bird populations: a historical study over three decades. *J. Appl. Ecol.* 39, 673–687. doi: 10.1046/j.1365-2664.2002.00745.x

Benton, T. G., Vickery, J. A., and Wilson, J. D. (2003). Farmland biodiversity: is habitat heterogeneity the key? *TREE* 18, 182–188. doi: 10.1016/S0169-5347(03)00011-9

Carson, R. (1962). *Silent Spring*. Boston, MA: Houghton Miffin.

Clark, D. R. Jr., LaVal, R. K., and Swineford, D. M. (1978). Dieldrin-induced mortality in an endangered species, the gray bat (*Myotis griscescens*). *Science* 199, 1357–1359. doi: 10.1126/science.564550

Davy, C. M., Russo, D., and Fenton, M. B. (2007). Use of native woodlands and traditional olive groves by foraging bats on a Mediterranean island: consequences for conservation. *J. Zool.* 273, 397–405. doi: 10.1111/j.1469-7998.2007.00343.x

DeLange, H. J., Lahr, J., Van der Pol, J. C., Wessels, Y., and Faber, J. H. (2009). Ecological vulnerability in wildlife: an expert judgement and multicriteria analysis tool using ecological traits to assess relative impact of pollutants. *Environ. Toxicol. Chem.* 28, 2233–2240. doi: 10.1897/08-626.1

Dietz, C., von Helversen, O., and Nill, D. (2007). *Handbuch der Fledermäuse Europas und Nordwestafrikas*. Stuttgart: Kosmos Naturführer.

Eidels, R. R., Sparks, D. W., Whitaker, J. O., and Sprague, C. A. (2016). Sub-lethal effects of chlorpyrifos on big brown bats (Eptesicus fuscus). *Arch. Environ. Contam. Toxicol.* 71, 322–335. doi: 10.1007/s00244-016-0307-3

Eitminavichiute, I. S., Strazdiene, V., Kadyte, B. A., and Vanagas, J. J. (1982). The accumulation of benzophosphate in soil animals. *Pedobiologia (Jena)*. 24, 23–28.

European Food Safety Authority (2009). Guidance document on risk assessment for birds and mammals on request from EFSA. *EFSA J.* 7, 1438. doi: 10.2903/j.efsa.2009.1438

Frouz, J. (1999). Use of soil dwelling Diptera (Insecta, Diptera) as bioindicators: a review of ecological requirements and response to disturbance. *Agric. Ecosys. Environ*. 74, 167–186. doi: 10.1016/S0167-8809(99)00036-5

Fuentes-Montemayor, E., Goulson, D., and Park, K. J. (2011). Pipistrelle bats and their prey do not benefit from widely applied agri-environment management prescriptions. *Biol. Conserv*. 144, 2233–2246. doi: 10.1016/j.biocon.2011.05.015

Geiger, F., Bengtsson, J., Berendse, F., Weisser, W. W., Emmerson, M., Morales, M. B., et al. (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic Appl. Ecol*. 11, 97–105. doi: 10.1016/j.baae.2009.12.001

Gelusco, K. N., Altenbach, J. S., and Wilson, D. E. (1976). Bat mortality: pesticide poisoning and migratory stress. *Science* 194, 184–186. doi: 10.1126/science.959845

Hahn, M., Brühl, C. A., and Stahlschmidt, P. (2017). Nocturnal insects in different crops and the potential food provision for bat species. *Mainzer Naturwissenschaftliches Archiv*. 53, 5–20.

Hayes, J. P. (2000). Assumptions and practical considerations in the design and interpretation of echolocation-monitoring studies. *Acta Chiropterologica* 2, 225–236.

Heim, O., Schröder, A., Eccard, J., Jung, K., and Voigt, C. C. (2016). Seasonal activity patterns of European bats above intensively used farmland. *Agric. Ecosyst. Environ.* 233, 130–139. doi: 10.1016/j.agee.2016.09.002

Hofmann, K. (1991). Vergiftung junger mausohren (*Myotis myotis*) durch Pflanzenschutzmittel. *Nyctalus* 4, 85–87.

Jefferies, D. J. (1972). Organochlorine insecticide residue in British bats and their significance. *J. Zool. Lond.* 166, 245–263. doi: 10.1111/j.1469-7998.1972.tb04088.x

Kalda, O., Kalda, R., and Liira, J. (2014). Multi-scale ecology of insectivorous bats in agricltural landscapes. *Agric. Ecosyst. Environ*. 199, 105–113. doi: 10.1016/j.agee.2014.08.028

König, H., and Wissing, H. (2007). *Die Fledermäuse der Pfalz*. Mainz: GNOR.

Krebs, J. R., Wilson, J. D., Bradbury, R. B., and Siriwardena, G. M. (1999). The second silent spring? *Nature* 400, 611–612. doi: 10.1038/23127

Morris, A. D., Miller, D. A., and Kalcounis-Rueppell, M. C. (2010). Use of forest edges by bats in a managed pine forest landscape. *J. Wildlife Manag*. 74, 26–34. doi: 10.2193/2008-471

Nielsen, B. E., Nielsen, B. L., Axelsen, J., and Elmegaard, N. (1994). Winter abundance of soil *Diptera* larvae in arable soil. *Pedobiologica* 38, 208–221.

O'Shea, T. J., and Johnson, J. J. (2009). "Environmental contaminants and bats: investigating exposure and effects," in *Ecological and Behavioral Methods for the Study of Bats*, *2nd Edn*., eds T. H. Kunz and S. Parsons (Baltimore: Johns Hopkins University Press), 500–528.

Park, K. J. (2015). Mitigating the impacts of agriculture on biodiversity: bats and their potential role a bioindicators. *Mamm. Biol*. 80, 191–204. doi: 10.1016/j.mambio.2014.10.004

Park, K. J., Müller, C. T., Markman, S., Swinscow-Hall, O., Pascoe, D., and Buchanan, K. L. (2009). Detection of endocrine disrupting chemicals in aerial invertebrates at sewage treatment works. *Chemosphere* 77, 1459–1464. doi: 10.1016/j.chemosphere.2009.08.063

Racey, P. A., and Swift, S. M. (1985). Feeding ecology of *Pipistrellus pipistrellus* (Chiroptera: Vespertilionidae) during pregnancy and lactation. I. Foraging behaviour. *J. Appl. Ecol*. 54, 205–215. doi: 10.2307/4631

Roßberg, D. (2007). NEPTUN oder "Wie oft wird gespritzt." *Gesunde Pflanze* 59, 55–65. doi: 10.1007/s10343-007-0146-2

Roßberg, D., and Harzer, U. (2015). Survey on application of chemical pesticides in apple farming. *J. Kulturpflanzen* 67, 85–91. doi: 10.5073/JfK.2015.03.01

Runkel, V. (2008). *Mikrohabitatnutzung Syntoper Waldfledermäuse*. Ph.D. thesis. Universität Erlangen-Nürnberg.

Russo, D., and Jones, G. (2003). Use of foraging habitats by bats in a Mediterranean area determined by acoustic surveys: conservation implications. *Ecography* 26, 197–209. doi: 10.1034/j.1600-0587.2003.03422.x

Skiba, R. (2009). *Europäische Fledermäuse-Kennzeichen, Echoortung und Detektoran-Wedung. Die neue Brehm-Bücherei*. Westarp Wissenschaften-Verlagsgesellschaft Hohenwarsleben. Magdeburg: VerlagsKG Wolf.

Stahlschmidt, P., and Brühl, C. A. (2012a). Bats as bioindicators–the need of a standardized method for acoustic bat activity surveys. *Methods Ecol. Evol*. 3, 503–508. doi: 10.1111/j.2041-210X.2012.00188.x

Stahlschmidt, P., and Brühl, C. A. (2012b). Bats at risk? Bat activity and insecticide residue analysis of food items in an apple orchard. *Environ. Toxicol. Chem*. 31, 1556–1563. doi: 10.1002/etc.1834

Stahlschmidt, P., Pätzold, A., Ressl, L., Schulz, R., and Brühl, C. A. (2012). Constructed wetlands support bats in agricultural landscapes. *Basic Appl. Ecol*. 13, 196–203. doi: 10.1016/j.baae.2012.02.001

Vaughan, N., Jones, G., and Harris, S. (1997). Habitat use by bats (*Chiroptera*) assessed by the means of a broad-band acoustic method. *J. Appl. Ecol*. 34, 716–730. doi: 10.2307/2404918

Walsh, A. L., and Harris, S. (1996). Foraging habitat preferences of vespertilionid bats in Britain. *J. Appl. Ecol*. 33, 508–518. doi: 10.2307/2404980

Wickramasinghe, L. P., Harris, S., Jones, G., and Jennings, N. V. (2004). Abundance and species richness of nocturnal insects on organic and conventional farms: effects of agricultural intensification on bat foraging. *Conserv. Biol*. 18, 1283–1292. doi: 10.1111/j.1523-1739.2004.00152.x

Wickramasinghe, L. P., Harris, S., Jones, G., and Vaughan, N. (2003). Bat activity and species richness on organic and conventional farms: impact of agricultural intensification. *J. Appl. Ecol*. 40, 984–993. doi: 10.1111/j.1365-2664.2003.00856.x

Zehetmair, T., Müller, J., Runkel, V., Stahlschmidt, P., Winter, S., Zharov, A., et al. (2015). Poor effectiveness of Natura 2000 beech forests in protecting forest-dwelling bats. *J. Nat. Conserv*. 23, 53–60. doi: 10.1016/j.jnc.2014.07.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Stahlschmidt, Hahn and Brühl. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 19 December 2016
doi: 10.3389/fenvs.2016.00080

Modeling Exposure of Mammalian Predators to Anticoagulant Rodenticides

*Christopher J. Topping * and Morten Elmeros*

Department of Bioscience, Aarhus University, Rønde, Denmark

Anticoagulant rodenticides (AR) are a widespread and effective method of rodent control but there is concern about the impact these may have on non-target organisms, in particular secondary poisoning of rodent predators. Incidence and concentration of AR in free-living predators in Denmark is very high. We postulate that this is caused by widespread exposure due to widespread use of AR in Denmark in and around buildings. To investigate this theory a spatio-temporal model of AR use and mammalian predator distribution was created. This model was supported by data from an experimental study of mice as vectors of AR, and was used to evaluate likely impacts of restrictions imposed on AR use in Denmark banning the use of rodenticides for plant protection in woodlands and tree-crops. The model uses input based on frequencies and timings of baiting for rodent control for urban, rural and woodland locations and creates an exposure map based on spatio-temporal modeling of movement of mice-vectored AR (based on *Apodemus flavicollis*). Simulated predator territories were super-imposed over this exposure map to create an exposure index. Predictions from the model concur with field studies of AR prevalence both before and after the change in AR use. In most cases, incidence of exposure to AR is predicted to be <90%, although cessation of use in woodlots and Christmas tree plantations should reduce mean exposure concentrations. Model results suggest that the driver of high AR incidence in non-target small mammal predators is likely to be the pattern of use and not the distance AR is vectored. Reducing baiting frequency by 75% had different effects depending on the landscape simulated, but having a maximum of 12% reduction in exposure incidence, and in one landscape a maximum reduction of <2%. We discuss sources of uncertainty in the model and directions for future development of predictive models for environmental impact assessment of rodenticides. The majority of model assumptions and uncertainties err on the side of reducing the exposure index, hence we believe the predictions to be robust and to indicate that the scale of the problem may be large.

Keywords: anti-coagulants, secondary poisoning, spatial modeling, ALMaSS, mustelid home-ranges, mouse dispersal

OPEN ACCESS

Edited by:
Carsten A. Brühl, University of Koblenz and Landau, Germany

Reviewed by:
Hanna Weise, Freie Universität Berlin, Germany
Richard Shore, Centre for Ecology & Hydrology (CEH), UK

****Correspondence:***
Christopher J. Topping
cjt@bios.au.dk

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *12 August 2016*
Accepted: *01 December 2016*
Published: *19 December 2016*

Citation:
Topping CJ and Elmeros M (2016) Modeling Exposure of Mammalian Predators to Anticoagulant Rodenticides. Front. Environ. Sci. 4:80. doi: 10.3389/fenvs.2016.00080

INTRODUCTION

Anticoagulant rodenticides (AR) are a widespread and effective method of rodent control (WHO, 1995; Erickson and Urban, 2004; Laakso et al., 2010). AR are slow-acting toxins that block vitamin K regulating blood's ability to clot. The poisoned animals typically die as a result of internal bleeding 8–10 days after ingesting a lethal dose of poison. The development of resistance to the poisons (e.g., warfarin and coumatetralyl) in rodents led to the development of the more toxic "second-generation" AR (Pelz, 2001; Lodal, 2010; Vein et al., 2011). These second-generation compounds are also metabolized and excreted more slowly than their predecessors. They tend to accumulate in liver and can have very long internal half-lives (e.g., 307 days for brodifacoum (Vandenbroucke et al., 2008). These second-generation ARs therefore pose an increased risk of secondary poisoning of small mammal predators (Alterio, 1996; Berny et al., 1997; Newton et al., 1999; Erickson and Urban, 2004; Dowding et al., 2010; Laakso et al., 2010). In fact the risk of secondary poisoning is well known and was described for first generation compounds in the 1960s (Evans and Ward, 1967). Subsequently, studies in the laboratory and the field have documented lethal effects of secondary exposure to AR in predators (Alterio et al., 1997; Berny et al., 1997; Murphy et al., 1998; Giraudoux et al., 2006; Dowding et al., 2010; Walker et al., 2010).

Small-mammal predators (mustelids and birds) assayed for exposure to AR in Denmark have an incidence of exposure of 84–100% depending upon species (Elmeros et al., 2011; Christensen et al., 2012). Not only was the percentage of animals exposed high in the Danish predators, but in most species there were individuals (up to 70%) with rodenticide concentrations in the liver above (200 ng/g live-weight); a potentially lethal concentration for mustelids and birds of prey (Grolleau et al., 1989; Newton et al., 1999). Some predators also had concentrations that are associated with lethal toxic effects in the fox, 800 ng/g live-weight (Berny et al., 1997).

Rodenticide concentration levels in weasels and stoats suggests Danish animals have a higher occurrence of positives and higher concentrations in Denmark than found in other European countries (Shore et al., 2003; Elmeros et al., 2011), although this to a certain may be accounted for by differential sensitivity of the analytical techniques. High rates and concentrations of AR in predators have similarly been found in France and New Zealand in association with intensive rodent control campaigns (Alterio, 1996; Alterio et al., 1997; Berny et al., 1997; Murphy et al., 1998; Eason et al., 1999).

The cause of the very high rates of AR in non-target predators in Denmark is not known. The predators assayed previously were collected during a period when the use of AR to combat rodents in forests, tree crops, and game feeding stations was allowed (Elmeros et al., 2011; Christensen et al., 2012). In 2010, the Danish EPA changed the regulations and prevented use of rodenticides in woodland and tree-crops by the end of 2011. The aim was in part to reduce the prevalence of rodenticide in non-target organisms, and was based on the assumption that use in these habitats conferred the highest risk of exposure (i.e., contact between the non-target animal and the rodenticide). However, a recent assay found that there was no decrease in prevalence of AR in either stone marten or polecat. On the contrary, the AR burden in stone marten increased from 1999–2004 to 2012–2013, while it remained constant in polecats (Elmeros et al., 2015).

In this study we consider two possible hypotheses to explain the almost universal exposure of predators in Denmark. The first was that rodent vectored rodenticide might spread far enough to become available to the majority of predators; the second being that the pattern of household and urban use might be enough in itself to explain the intensive secondary exposure of predators. We also consider whether the regulation changes in 2010 should have been enough to reduce exposure of non-targets significantly by removing rodenticides from woodlands and other uses away from buildings.

The primary focus of this study was the development of model representing the coincidence of predators and rodenticide in time and space. However, to support the model a study of how far mice might vector rodenticide using mark-release-recapture was also undertaken. The modeling tool included dynamic modeling of the fate of rodenticide, baiting frequency, timing and placement, and estimation of predator territory density and locations at 1-m^2 resolution on three 10 × 10-km landscapes. Finally, the real-world impact of new Danish regulations was evaluated using a new survey of rodenticide concentrations found in Danish mammalian small-mammal predators (Elmeros et al., 2015), post the new rodenticide restrictions imposed by the Danish EPA. This data was also used to compare to model predictions.

MATERIALS AND METHODS

Capture-Mark-Recapture

To elucidate on the potential rodent vectored rodenticide dispersal away from baiting sites to become available in a larger area to the majority of small mammal predators, we determined dispersal distances of small rodents during the autumn, when rodenticide use is most intensive in Denmark.

Dispersal distances of anticoagulant rodenticide poisoned mice and voles from a bait box were assessed by capture-mark-recapture over an 8 week period in two study areas during the autumn (mid-September–mid-November) 2012. Rodents were caught in Ugglan multiple-capture live-traps. The traps were baited with ample food (apples and unhusked seeds) and supplied hay as bedding material and checked once a day. Traps were positioned on the ground sheltered by vegetation, i.e., protected from extreme weather. A solid lid on the traps provided cover from exposure to precipitation and wind. The two study areas (75 × 200 m) comprised patches of herbs, shrubs, and trees. The areas were delimited by local landscape features and contained 147 and 149 traps, respectively. Within each study area the traps were evenly distributed 15 × 15 m apart around each baiting station. Individual rodents weighing >15 g were marked subcutaneously with a small PIT-tag (Passive Integrated Transponder, Oregon RFID). Individuals below 15 g were not tagged on ethical grounds. Hence, only dispersal distances for rodents >15 g were recorded. Mice and voles were trapped and marked during two 5-day trapping sessions with 2 week intervals and a final trapping session in the 7th and 8th week.

Dispersal distances between captures (all inter-trap distances) of small mammals were compared between species and study areas by generalized linear mixed modeling.

ALMaSS

The model was developed within ALMaSS (Topping et al., 2003), an agent-based simulation system utilizing a highly configurable and detailed landscape model component (Topping et al., 2016). ALMaSS is an open source C++ project, and all code is available through the GitLab[1]. In addition, documented code using ODdox documentation for the rodenticide model is available via an internet link[2]. The rodenticide model was created partly utilizing the existing landscape sub-model which was extended to handle baiting location coding and rodenticide baiting timing and frequencies (see below). The complete model was developed by creating two further sub-models to enable rodenticide handling within the landscape (represented by the *Rodenticide* and the *RodenticidePredator* C++ classes in the program). These classes implemented all other behavior necessary for the rodenticide model (see ODdox[2]). The overall modeling strategy was somewhat similar to (e.g., Nogeire et al., 2015) in that we attempted to create distributions of predators based on habitat structure and then overlay exposure. The main differences were that we had no predator population dynamics, the scale which is much smaller in our study and more detailed, and that we used a more detailed simulation of baiting patterns, and subsequent AR vectoring and environmental decay.

Landscape Maps

To evaluate the variability associated with landscape structure, all scenarios were run on three different 10 × 10 km landscapes with differing compositions representing real rural Danish landscapes (see Supplementary Material). These landscapes represent a small field, locally heterogeneous regionally homogenous landscape (Herning), a typical estate landscape with large fields and large wooded areas (Præstø), and an intermediate landscape (Bjerringbro).

Mouse-Vectored Rodenticide Simulation

Rates of mouse dispersal when vectoring rodenticide were assumed to be the same as dispersal rates measured from the capture-mark-recapture study (see Results section). Rates of dispersal were somewhat varied for the different species of rodent. However, maximum distance moved only varied little between species. The dispersal rates were considered as a diffusion process from a point source. Since variation in maximum distance moved between the three sets of curves was small only the yellow-necked mouse data was used for fitting and subsequent scenarios. To fit this diffusion process to the 2-dimensional representation required by the model, the curve of frequency of distance moved was used to calibrate the model procedure.

The equation used to model diffusion evaluates the amount of rodenticide present in a single cell at a time. It is assumed that diffusion processes in all directions operate equally and that at each time step of one day a fixed proportion (p) of the rodenticide present (a_t) is distributed to all surrounding cells. The rate and distance of travel is also determined by two other parameters, d is the cell width in meters, and D is the assumed to be the rate of decay resulting in a half-life of the rodenticide after bait placement. This is primarily meant to represent an internal half-life once ingested by mice, but will also operate on the baiting location to remove rodenticide assumed to be as a result of other routes (e.g., rats, slugs). At each time step the amount of rodenticide leaving a cell (a_o) is given by Equation (1). This amount is divided equally between all cells of size d bordering the current cell.

$$a_o = (a_t(1 - D))p \tag{1}$$

The amount of rodenticide entering a cell is given by the sum of the amounts leaving all surrounding cells. The change in rodenticide amount per cell is given by Equation (2).

$$a_{t+1} = \sum_{k=0}^{n} a_{ok} + a_t - a_o \tag{2}$$

where k is the number of surrounding cells.

Parameters p and d were varied and used to calibrate the diffusion model to fit as closely as possible to the mouse dispersal data. Evaluation of fit was by using a least squares estimation resulting in three sets of parameters. Fits were reasonably good, although not perfect (**Figure 1**). In all cases the fit was made ignoring the 0–10 m category, since the step-wise nature of the model introduced excessive noise when diffusion to so few cells was included, especially since cell sizes of 5 × 5 m were found to provide the best fits.

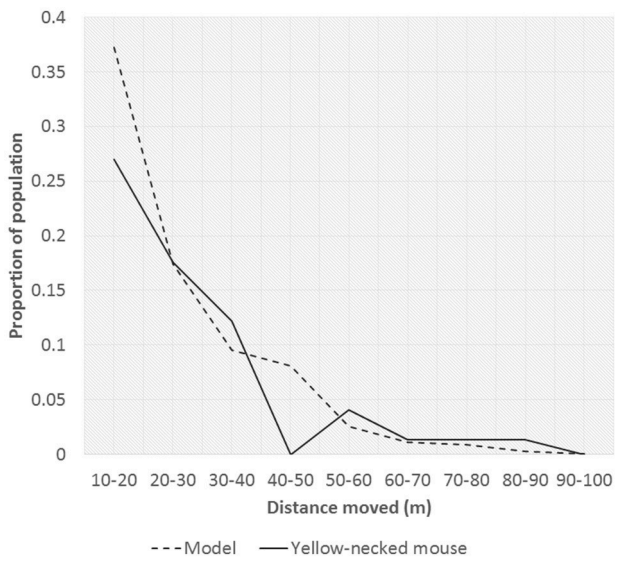


FIGURE 1 | Yellow-necked mouse dispersal distances and fitted diffusion model.

[1]https://gitlab.com/groups/ALMaSS

[2]https://almassdocs.au.dk/ALMaSSODdox/RodenticideModel/index.html

Landscape Coding and Bait Locations

To determine the bait locations from a landscape map, the map features were classified into types of potential bait locations and other features. The ALMaSS map is polygon-based, and as such each element is represented by a polygon and classified into 55 element types. Potential bait locations were determined to be buildings, woodlands, and Christmas tree plantations. These elements were identified and a central point lying inside each polygon found by calculating the center of the minimum sized rectangle which could surround the polygon and then searching outward in a spiral pattern from this point until a location was found inside the polygon in question. The spiraling was only needed in the cases where polygons had other areas within them (e.g., doughnut shaped).

Buildings required a further step to classify them as urban or country buildings since these have different baiting profiles. All buildings were initially designated as urban. Then the minimum distance to the nearest 6 other buildings was calculated for each building. The building was reclassified as country if this minimum distance exceeded 100-m. The values of 100-m and 6 buildings were determined by visual inspection of the resulting classification on the Bjerringbro map before fixing these values for all simulations.

Each baiting location was designated a type based on the polygon type, thus four categories of baiting location were created (woodland, Christmas trees, country buildings, and urban building). Each baiting location also had type-dependent annual probability of bait placement (i.e., the annual chance that bait is used at all at this location), and a seasonal distribution of likely bait placement dates (i.e., a probability based distribution of date of placement if used). During a simulation the annual bait placement probability was used at the beginning of each year to determine whether bait was placed at that point in that year—if so the date was selected from a type-specific list of potential baiting dates. The list of dates comprised of a set of 365 daily probabilities summing to 1.0. Seasonal baiting could therefore be simulated by weighting or zeroing probabilities of bait placement for individual dates or periods. Once bait was placed it was assumed to be present at the point source for a type-specific period; these periods being determined by estimates of treatment times from professional pest-control practitioners (see Scenario Development below). Once a bait location had been active for the designated period it was assumed to be used up or removed and thus no further bait would be added resulting in a slow degradation of bait the rate dependent upon the half-life parameter D (see Equation 1).

Predator Modeling

Predator modeling consisted of two separate processes: The first was to define predator territories where exposure to rodenticide could be evaluated. The second stage was collection of exposure information during the scenario runs.

Defining predator territories: Predator home-ranges (H-R) were assumed to be square. Determination of a territory was calculated separately for each predator type by the model based on three criteria:

TABLE 1 | Typical home-range (H-R) sizes of mustelids in Denmark based on European studies (see Supplementary Table 2).

Species		Range of home-range size (ha)	
		Males	Females
Pine marten	*Martes martes*	200–500	100–300
Stone marten	*Martes foina*	200–500	100–300
Polecat	*Mustela putorius*	300–750	10–300
Stoat	*Mustela erminea*	50–250	30–150
Weasel	*Mustela nivalis*	50–300	25–100

1) That the sum of the scores of the areas contained within the H-R exceeded a minimum level, representing the habitat resources needed by each species. The H-R score was built up of individual scores for each 1-m^2 or each habitat within the territory. The habitat score was based on the landscape element type indexing a species specific set of landscape element type scores (e.g., a stone marten will score a building higher than a pine marten). The value of the scores were determined by expert judgment and fitting of the territories to the Bjerringbro landscape. These scores were then also used for the Præstø and Herning landscapes to generate predator territory maps.
2) That the home-range size achieving the score was between a maximum and minimum size (measured as width), and based on literature estimates for each species (**Table 1**);
3) That the territory did not overlap with another predator territory;

Predator territories were created for each map once, and stored for future use in all scenarios. Hence, it is possible to directly compare exposure between landscapes within a species. The method of territory creation was to start randomly within one maximum territory distance, but not less than half the territory width, from the NW corner of the map and evaluate whether a minimum sized territory centered on that location fulfilled the above criteria. If so the territory was placed and the map area claimed (could not be used for new territories). If it was not possible to create a territory the territory size was incremented the procedure repeated until either maximum size was reached or it was possible to place the territory. At this point the next map location to the east was selected and the procedure repeated. If there were no more locations to the east then the first location on the row one south was selected and the procedure repeated. Once all locations had been evaluated then the procedure was halted and the map finalized. Example territory locations are shown in **Figure 2**. Each time this procedure was applied slightly different maps were generated, but initial testing indicated that the variation due to this factor was negligible.

Evaluating predatory territory exposure: This was achieved by creating an index of exposure based on the mean daily total amount of mouse-vectored rodenticide present in the territory. Each day during the simulation every 1 × 1-m grid cell was evaluated for rodenticide exposure and the total summed for the whole territory. At the end of the simulation the total was divided by the number of days counted and saved as output. This statistic

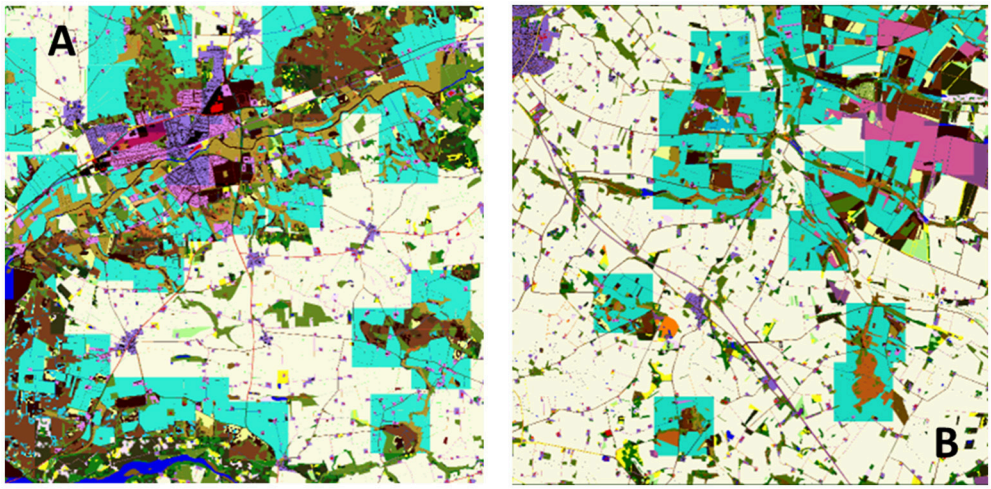


FIGURE 2 | Example predator territory locations (overlay in blue) in two 10 × 10 km landscape maps, Bjerringbro (A) and Herning **(B)** for *M. nivalis*. In this case territories are clustered around semi-natural landscape elements and avoid town areas and open country. The territories are represented as squares of a minimum size required to achieve the minimum territory score based on the area of underlying habitats.

is not real exposure, but an index of the available rodenticide in a territory, hereafter referred to as an "exposure index."

Scenario Development

There was no available systematic survey on the frequency and duration of treatment nor the between year variation in the pattern of rodent control in Denmark. The values for the annual frequency, time of year and the duration of baiting in different landscape elements included in the model were determined from interviews and assessments with professional pest control, rat consultants, and former users of rodenticides in forestry and Christmas tree production. Information on length of baiting period was obtained in the form of x% for 1 month, y% for 2 months, and z% for 6 months. The mean length of baiting was therefore calculated as:

$$days = (30x + 60y + 180z)/100 \quad (3)$$

Default frequency, length and timing of baiting is given by **Table 2**. All scenarios were run on all three landscapes, with a default half-life of 14-days unless otherwise specified below. In all cases 100 replicates of 1 year of each scenario were run, enough to ensure mean exposures were stable (within 1%) on addition of further replicates. In all cases, the likelihood of baiting a location (e.g., an urban building) in the model depends firstly on the annual by area probability (5%) for an urban building. If bating will occur then there is a probability applied determining the time of year it is baited, and then the bait is simulated in the model for the baiting length from Equation (3).

TABLE 2 | The default frequency, timings of start of baiting and baiting duration used for the all scenarios unless otherwise specified.

Landscape element type	Frequency % by area per year	Timing of baiting	Baiting frequency of duration	Mean baiting period (days)
Urban Building	5	25% spring, 75% autumn	94%, 30 days; 5%, 60 days; 1%, 180 days	33
Country Building	33	25% spring, 75% autumn	94%, 30 days; 5%, 60 days; 1%, 180 days	33
Christmas Trees	2.8% (5% per year in years 2–6 of 9-year production)	September–October	75%, 30 days; 15%, 60 days; 10%, 180 days	44
Young Woodlot	0.1% (1% of forest replanted annually, and 10% of this baited).	July–October	75%, 60 days; 15%, 120 days; 10%, 180 days	50

All data based on information provided by pest-control consultants and foresters/growers.

Experiments

Experiment 1—Evaluation of Present and Past Exposure

The main set of simulations was carried out for five species (*Martes martes, Martes foina, Mustela putorius, Mustela ermine, Mustela nivalis*), on all three landscapes and consisted of two scenarios. Scenario 1 (baseline) assumed the standard situation prior to 2012, where pesticides were used in wooded areas and Christmas trees. Scenario 2 (present day), assumed the same distribution and frequency of baiting locations for buildings, but zero rodenticide usage in Christmas trees and woodlots. In all scenarios the half-life of rodenticide exposure was set to be 14 days and frequency and timing of baiting set to the standard conditions described by the consultants.

Experiment 2—Sensitivity to Environmental Half-Life

The half-life parameter is not as simple as a normal environmental half-life since it covers the availability of the

rodenticide to the predators. This includes the concentration in mice before they die, and any storage of contaminated food which may be eaten later, as well as whether dead mice may be consumed. Half-life in the mice (e.g., of bromadiolone) is 28 days (Vandenbroucke et al., 2008), with other typical 2nd generation rodenticides varying between 16 and 307 days. For all main scenarios we chose a half-life of 14 days (conservative for estimating exposure), but carried out a sensitivity analysis for two species (*M. foina* and *M. nivalis*) varying half-life to be 7, 14, 28, and 56 days.

Experiment 3—Baiting Frequency

One of the largest uncertainties present in determining the exposure is the frequency of baiting. Therefore, a third experiment was run to determine the sensitivity of the results to the frequency of baiting. Two additional baiting frequencies were considered for *M. foina* and *M. nivalis*, at 50 and 25% the frequency suggested by the consultants.

Analysis

Analysis was performed on the potential rodenticide exposure index as calculated by each predator territory. Means were calculated for each scenario from all predator territories present in the landscape (between 10 and 40 depending upon species and landscape). All statistics were calculated by pooling all 100 replicates for each species used. The number of territories that were unexposed (exposure index = 0.0) was also recorded and the proportion of these calculated. These indicate zero exposure within 1 year, but do not necessarily mean that the territory was not exposed between years.

RESULTS

Mark Release Recapture

A total of 220 bank voles (*Myodes glareolus*), 63 field voles (*Microtus agrestis*) and 66 yellow-necked mice (*Apodemus flavicollis*) were marked, and 561, 77, and 74 recaptures were recorded. Mean dispersal distances between recaptures of yellow-necked mice, bank voles and field voles were 20.2 ± 18.0 m, 11.2 ± 13.2 m, and 9.0 ± 10.7 m (**Table 3**). Yellow-necked mice dispersed further than the other species (**Table 4**).

Experiment 1: Evaluation of Present and Past Exposure

The major result of these simulations is that a very low percentage of predator territories have zero exposure to rodenticides. The cessation of use of rodenticides in woodland and Christmas tree areas is predicted to have an effect on the total exposure index, but only a very minor impact on the proportion of territories unexposed (**Table 5**). The highest proportion of unexposed territories was 3.14% in Herning for *M. nivalis*, but across all cessation scenarios there was a mean of 0.29% and median of 0.00%. **Figure 3** shows a map of the exposure index in the Bjerringbro landscape in late September, when rodenticide levels are at the peak. There is a concentration in the town area, but very few areas are not exposed across the landscape.

Mean exposure index values differed markedly between species as a function of location and size of territory, with highest exposure in polecat (*M. putoris*) and lowest in weasel (*M. nivalis*). This is as expected simply due to differences in H-R sizes, but there was also a sizeable variation between landscapes, with exposure values for Bjerringbro being 3–4 times higher than Herning and Præstø. Cessation of woodland and Christmas tree treatment reduced mean exposure index scores by 15–37% with highest reductions in the weasel, and lowest reductions on average in the polecat.

Coefficient of variation increases noticeably on cessation of woodland use of rodenticides. This is in keeping with the higher variability expected, especially in the predators with smaller territories and less association with human habitation (e.g., stoat and weasel), but impacts were seen for all species. This suggests that although still exposed there was a greater variation in

TABLE 4 | Relationship between dispersal distances of marked mice and voles, species, study areas and trapping sessions as determined by GLMM.

Variable	*d.f.*	*F*	*P*	Differences
Species	2	3.93	0.02	Yellow-necked mouse > bank vole, field vole
Study area	1	0.32	0.57	–
During / between trapping weeks	1	0.12	0.72	–
Species*study area	2	0.80	0.45	–

TABLE 3 | Summary of dispersal distances of marked mice and voles over 8 weeks during autumn 2012.

Study area	Species	Marked individuals	Re-captured individuals	Re-captures	Dispersal distances (m)	
					Mean ± SD	Min.–Max.
A	Bank vole	116	96	281	13.1 ± 16.6	0–175.9
	Field vole	16	7	11	8.5 ± 6.2	0–20.0
	Yellow-necked mouse	34	20	38	17.1 ± 16.6	0–73.6
B	Bank vole	104	85	280	9.2 ± 8.4	0–44.8
	Field vole	47	32	66	9.0 ± 11.4	0–51.2
	Yellow-necked mouse	32	20	36	23.6 ± 19.1	0–86.7

TABLE 5 | Results of Experiment 1 for the each of the three landscapes as exposure indices.

		Martes martes	*Martes foina*	*Mustela putorius*	*Mustela erminea*	*Mustela nivalis*
Bjerringbro						
Baseline	Mean	49511	43663	57827	25170	20149
	Min	4751	5493	3895	430	595
	Max	158110	155794	212466	143658	120494
	Not Exposed	0.00%	0.00%	0.00%	0.00%	0.00%
Present	Mean	37806	33767	45173	18419	14653
	Min	4702	943	2860	39	4
	Max	139772	132325	204496	125766	110999
	Not Exposed	0.00%	0.00%	0.00%	0.00%	0.00%
	Exposure Reduction	23.64%	22.66%	21.88%	26.82%	27.28%
Herning						
Baseline	Mean	11889	12791	18645	7582	4944
	Min	42	510	584	0	0
	Max	44763	40529	44628	29765	18103
	Not Exposed	0.00%	0.00%	0.00%	0.05%	0.24%
Present	Mean	9505	10056	15786	5955	3529
	Min	1	1	260	0	0
	Max	35923	36800	40948	28528	14550
	Not Exposed	0.00%	0.00%	0.00%	0.67%	3.14%
	Exposure Reduction	20.05%	21.38%	15.33%	21.46%	28.63%
Præstø						
Baseline	Mean	11887	14444	18710	7752	6003
	Min	36	17	1081	5	0
	Max	44850	56486	50079	29081	26372
	Not Exposed	0.00%	0.00%	0.00%	0.00%	0.00%
Present	Mean	8451	10809	13605	4967	3789
	Min	38	7	291	0	0
	Max	30182	50518	36061	18059	16385
	Not Exposed	0.00%	0.00%	0.00%	0.04%	0.53%
	Exposure Reduction	28.91%	25.17%	27.28%	35.93%	36.88%

Exposure reduction is the percentage change in the Exposure Index from the Baseline conditions (prior to 2011) to the present day scenario.

exposure following cessation, indicating a positive impact greater than suggested by the reduced percentage of territories with zero exposure.

Experiment 2: Sensitivity to Environmental Half-Life

Increasing or decreasing half-life increased or decreased the exposure index close to proportionally, a second order polynomial having a perfect fit to the data (e.g., Bjerringbro *M. foina* $y = -7.0655x2 + 1192.4x - 720.89$, $R^2 = 1$). However, even with a 7-day half-life, the proportion of territories unexposed was very low, close to zero for *M. foina* and between zero, and 5% for *M. nivalis*, depending upon the landscape (**Table 6**).

Experiment 3: Baiting Frequency

Altering the assumptions related to the baiting frequency by reducing the frequency by 50 and 75% reduced the mean index of exposure linearly with the reduction in baiting frequency for both *M. foina* and *M. nivalis* (**Figure 4**). The rate of reduction was different in Bjerringbro compared to Herning and Præstø due to the higher predicted exposure in that landscape. Reductions in the number of territories not exposed were also predicted but these were not linear and for *M. foina* reductions of 75% were needed to achieve increases in unexposed territories of just over 0.5% in Bjerringbro, with maximum reductions of 4% in Herning. Præstø was intermediate between these two; for *M. nivalis* the reductions were greater, but not proportionally so. Maximum reductions were also in Herning (almost 12%), but reductions in Præstø were only slightly lower. In Bjerringbro, even at 75% reduced baiting frequency more than 99% of weasel territories were exposed (**Figure 4**).

DISCUSSION

Model Uncertainties

The results of the overall modeling exercise are very clear. There are few if any predators moving around the Danish landscape that do not have the potential to be exposed to rodenticide even if rodenticides are only used for rat control in and around buildings. There are, however, a number of key uncertainties in

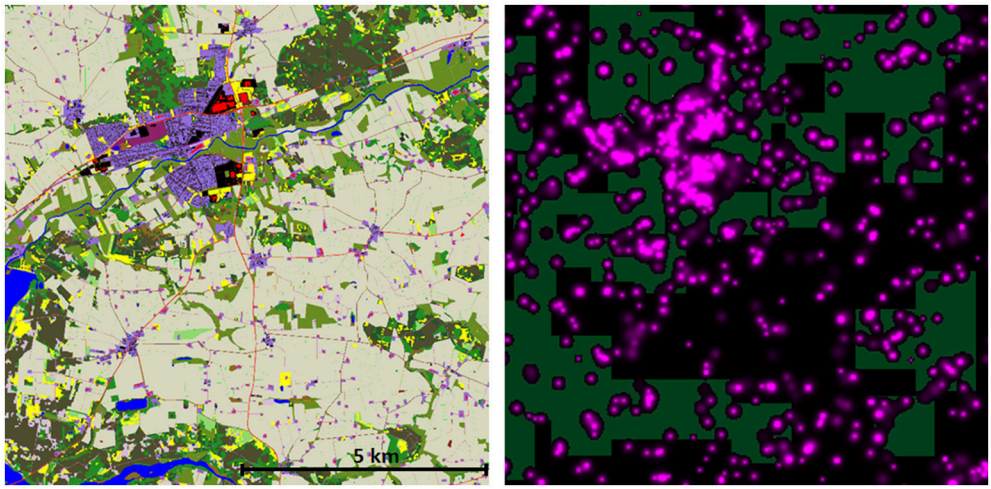


FIGURE 3 | Map of the Bjerringbro landscape (left) and an AR exposure map from September 30th for Scenario 2 (present day). Colored areas indicate modeled AC environmental concentration on this day. Note the distribution of AR does not allow for placement of the *M. nivalis* territories shown as light rectangles without covering one or more AR locations. See also Supplementary Material for a time-series of this data as a video showing the spatio-temporal dynamics of AR availability in the landscape.

the model to take into account, the main ones being the frequency of baiting, the patterns of rodenticide vectoring, the pattern of predator territories, and the extent to which potential exposure can be translated to real exposure. We have characterized these with respect to their effect on predicted impact.

Uncertainties that will Tend to Under-Estimate Impact with the Present Scenario:

- We have assumed only two types of baiting are present in the landscapes, urban and rural buildings. The frequency of baiting is therefore simplified to these two types, whereas in reality baiting may be more diverse (e.g., industrial units, sewers). The frequency may also be under or over estimated. Under estimation will not alter results significantly since results suggest that almost 100% of territories are exposed and this situation cannot be made much worse. If the frequency estimates are over-estimated then for weasels there may be an important effect in some landscapes. However, it does not seem likely that the professional pest control experts would make errors of the magnitude needed to create these effects (i.e., 400% too large), and even at this level of error very few territories will be unexposed (0–12% depending on species and landscape).
- There are also uncertainties regarding the distribution of rodenticide poisoned rodents. We assume it is vectored by mouse movement but take no account of changed mouse behavior which would under estimate exposure in the model if poisoned mice were easier to catch (Cox and Smith, 1992), and might affect the proportion of contaminated rodents in the diet. The diffusion model for rodent rodenticide dispersal compares well with recorded dispersal distances of poisoned

TABLE 6 | The effects of varying the environmental half-life parameter on exposure of *M. foina* and *M. nivalis* for three landscapes.

Landscape	Half-life	7d	14d	28d	56d
M. foina					
Bjerringbro	Mean	16661	33767	62410	101693
	Min	4	943	2063	2078
	Max	68029	132325	249603	428126
	Not Exposed	0.000%	0.000%	0.000%	0.000%
Herning	Mean	4943	10056	18578	30159
	Min	0	1	31	75
	Max	16319	36800	60779	97913
	Not Exposed	0.154%	0.000%	0.000%	0.000%
Præstø	Mean	5342	10809	19970	30159
	Min	0	7	28	75
	Max	23412	50518	93987	97913
	Not Exposed	0.000%	0.000%	0.000%	0.000%
M. nivalis					
Bjerringbro	Mean	7242	14653	27093	43898
	Min	0	4	71	137
	Max	55492	110999	202796	322624
	Not Exposed	0.025%	0.000%	0.000%	0.000%
Herning	Mean	1748	3529	6493	10583
	Min	0	0	0	0
	Max	7718	14550	26681	49378
	Not Exposed	5.048%	3.143%	0.286%	0.000%
Præstø	Mean	1844	3789	6962	11332
	Min	0	0	0	0
	Max	8346	16385	32593	46308
	Not Exposed	3.133%	0.533%	0.133%	0.033%

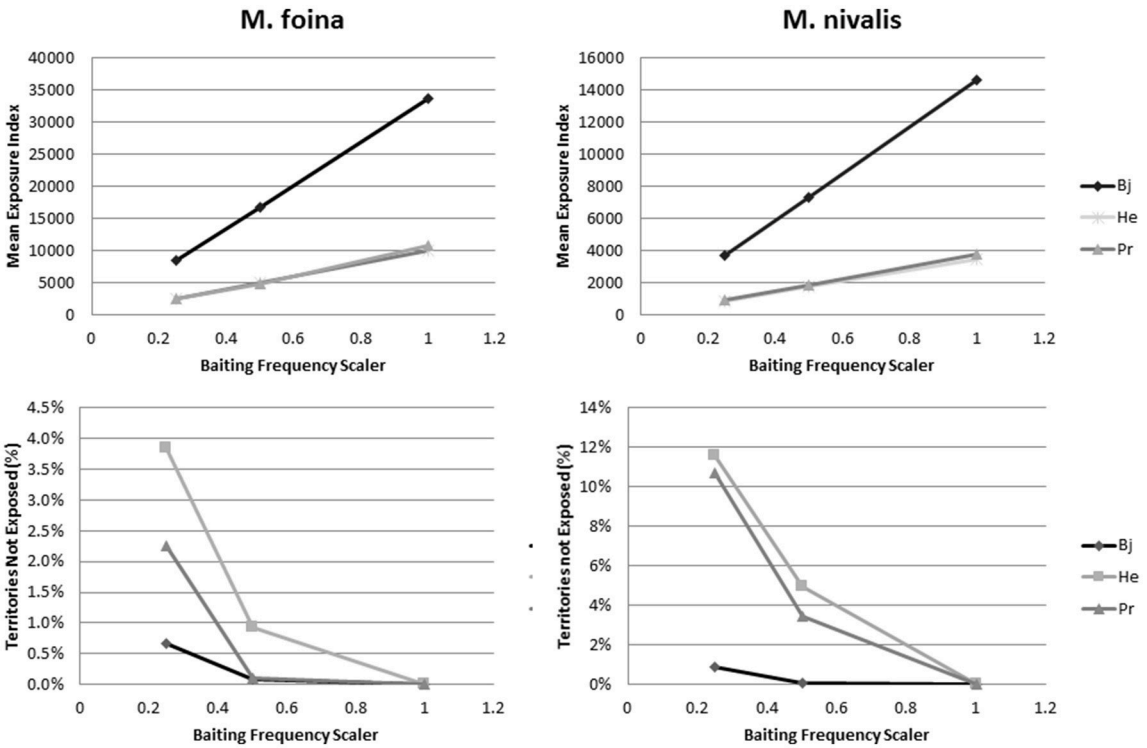


FIGURE 4 | Changes in mean exposure index and the percentage of territories not exposed when reducing the frequency of baiting to 50 and 75%. Bj, Bjerringbro; He, Herning; Pr, Præstø landscapes respectively. Each point is a based on multiple replicates, but with very small standard errors (not graphed).

small mammals during standard rat control operations with bait stations on farms (Geduhn et al., 2014). Dispersal distances of mice and voles are probably underestimated in these studies as individuals that moved away from the trapping grid were not recorded. Yellow-necked mice may disperse >1 km in one night (Stradiotto et al., 2009), i.e., potentially carrying poisoning long distance into the surrounding areas.

- We also assume 100% mouse and vole vectoring as these small rodents are the most dominant in the mustelid diet (Clevenger, 1994; Lode, 1997; Elmeros, 2006), but most poison is placed to control rats, and rural rats have much larger home-ranges and longer dispersal ranges than mice and voles (Taylor, 1978; Taylor and Quy, 1978; Lambert et al., 2008). It is also possible that bait is stored by rodents and is therefore available for a longer time than suggested (Jensen and Nielsen, 1986; McKenzie et al., 2005).
- The method of allocating predator territories has not been tested against real data and is based on expert judgment. However, real data on territories required to test the procedures used here is not available and would be difficult to obtain, thus there is little chance of immediate improvement of this part of the model. However, the modeled home-range sizes for the predators are probably underestimated and the fixed locations of H-R do not represent the variation in size and the high plasticity of mustelids foraging habitats.
- The model calculates exposure for a single year, and many predators are long-lived (e.g., maximum lifespan for free-ranging *M. foina* is >10 years (Ansorge and Jeschke, 1999), thus the life-time chance of exposure is higher than suggested here for the majority of species.

Uncertainties that will Tend to Over-Estimate Impact:

- The pattern of mouse deaths is not taken into account and may reduce rodenticide spread if it's faster than assumed (i.e., if mice die quickly), resulting in a shorter rodenticide environmental half-life than the 14 days used here. However, Experiment 2 suggested a low sensitivity to this parameter.
- Translation of the potential exposure predicted by the model to actual exposure is very difficult. Factors that come into play here are the efficiency of territory search and mouse capture, and the diet choice. For example the diet of stone martens is wider than mice (Clevenger, 1994), whereas weasel is a more specialized rodent predator (Elmeros, 2006). These difficulties mean that comparisons of exposure can only realistically be made within a species (relative exposure).
- We assume that all mice access the bait with equal probability. However, it has been shown that exposure in small mammal species varies in different UK landscapes, probably relating to habitat use and precise bait-box placement (Brakes and Smith, 2005). The direction of this bias is not certain, although, it probably over-estimates impact since some species and locations may reduce accessibility, whereas increasing it is not possible since the model assumes it is fully accessible.

In addition to these uncertainties, there is a general problem that the data against which we compare the model comes from a changing world where not all factors can be controlled. This can clearly be seen when we compare the results of reducing use of AR usage on stone martens, where AR burdens increased (Elmeros et al., 2015). Since there is no obvious mechanism for a decreased frequency and distribution of use to increase exposure it must be the real-world data that is inconsistent. This could be for many reasons, e.g., changing demography of the predators, changing spatial distribution, or most likely, a non-recorded change in baiting effort between the two measurements from the real world. In the case of stone marten and polecat this seems to have resulted in a an underestimate of effect in the model, but the actual effect cannot be known for sure without knowledge of which factors were changing the real world in which direction.

Implications of Results

Since the majority of the uncertainties are conservative, suggesting even higher levels of potential exposure than modeled, it seems reasonable to conclude that very few predators in the Danish landscape will have no chance of exposure to anti-coagulant rodenticide over the course of a year. This conclusion is strongly supported by the empirical evidence of the AR assays for small mammal predators carried out both pre- and post-regulation changes in Danish rodenticide use (Elmeros et al., 2015).

The actual exposure rates will vary with the landscape and species considered, with sparsely human—populated areas having lower exposure risk. But given that the three landscapes used here were typical of rural Denmark, then it is highly unlikely that large areas of the country are free of rodenticide, even after rodenticide use restrictions in woodlots. Cessation of rodenticide use in woodlots and Christmas trees and elsewhere away from building, e.g., game feeding stations has almost certainly reduced overall exposure rates considerably in wooded habitats, but has done little for exposure incidence. However, the assumption of standard baiting amounts may play a role and if significantly bias may alter this conclusion. There currently is no way of knowing whether the amounts of AR used per baiting application were the same in woodlots etc., as it was around buildings, as statistics on AR usage have not been compiled.

Exposure incidence is important because the method used here calculated a mean annual exposure, and thus it does not relate directly to dose. In this system dose is likely to vary considerably with time (Geduhn et al., 2016), since it is the chance encounter with an individual carrying a heavy rodenticide body burden that will determine maximum dose. Hence, toxico-dynamics are an important aspect to consider, and may mitigate against the decrease in Exposure Index if it is in fact AR use around buildings that typically provides these chance encounters.

Reducing the dispersal distances of mice had no significant impact on exposure estimates, and as such although of academic interest unless we consider very great variations in dispersal (e.g., kilometer scales, which would be the case for rats), then the precise form and slope of the dispersal function does not significantly affect exposure incidence in the model. Therefore, of our original two hypotheses explaining the almost universal exposure of predators in Denmark, it seems clear that the primary explanation is that the pattern of household and urban use might be enough in itself to explain the extent of secondary exposure of predators.

Future Model Development

The most important question to answer is what is the effect of exposure to rodenticides on populations of small mammal predators? To answer this requires three further steps.

Firstly we need to develop good models for the toxicological impacts on individuals as a result of receiving a dose of AR. There is little data on dose-response or even toxicity in non-target predators, and indications of very large variability in AR sensitivity in birds (Eason et al., 2002), and poor dose-response relationships in existing data (Rattner et al., 2014), but mammalian studies (e.g., Grolleau et al., 1989) are rare. Secondly, the estimates of exposure should be refined to relate model exposure to real world exposure. Whilst in our model the pattern of bait use is realistically represented, we do not know how this relates to transfer of ARs to predators. A quantitative estimate could be made by relating model predicted exposure correlated with real world body-burdens in space and time, e.g., using legally trapped pest species and road kills. Finally, these components need to be combined with spatio-temporal models of small mammal predator populations. Toxicity and exposure data could be combined to refine the simulation model, including toxico-dynamics, and toxico-kinetics to predict maximum dose. However, to function it will be necessary to map the spatial and temporal dynamics of both ARs in the environment and the predators. For example, there is seasonal variation in the use of farm buildings by polecats, where ARs are used (Birks, 1998), which may explain the increase in AR burdens in polecats in winter in Denmark (Elmeros et al., 2015). Such a population model should be individual-based (e.g., Nogeire et al., 2015), but should also ideally be able to represent environmental and behavioral details accurately to assess individual toxico-kinetics (e.g., Topping et al., 2016). A further improvement would be the use of multiple landscapes for testing scenarios. Recently the highly detailed landscapes used here have become easier to develop (at least for Denmark) (Topping et al, *loc.cit)* and thus new strategies could be evaluated against a wide range of real situations to evaluate their general applicability.

ETHICS STATEMENT

Trapping and tagging of rodents was done in accordance to a permit to trap and tag mammals and birds issued to Department of Bioscience by the Danish Nature Agency: SN 302-009 SEI.

AUTHOR CONTRIBUTIONS

Both CT and ME contributed to planning and execution of the work as well as to preparation of the manuscript.

FUNDING

The study was funded by research grants from the Danish Environmental Protection Agency (MST 667-00100 and MST 667-00112).

ACKNOWLEDGMENTS

Lene Jung Kjær, Jennifer Lynch, Jens Peder Hounisen, Lars Haugaard and Thomas K. Christensen provided valuable technical and field assistance. Peter Weile, the Nature Agency, and private PCOs are thanked for information on AR application patterns. Thanks also to the two reviewers for their helpful comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2016.00080/full#supplementary-material

REFERENCES

Alterio, N. (1996). Secondary poisoning of stoats (*Mustela erminea*), feral ferrets (*Mustela furo*), and feral house cats (*Felis catus*) by the anticoagulant poison, brodifacoum. *N. Z. J. Zool.* 23, 331–338. doi: 10.1080/03014223.1996.9518092

Alterio, N., Brown, K., and Moller, H. (1997). Secondary poisoning of mustelids in a New Zealand Nothofagus forest. *J. Zool.* 243, 863–869. doi: 10.1111/j.1469-7998.1997.tb01986.x

Ansorge, H., and Jeschke, D. (1999). Altersstruktur und Reproduktion des Steinmarders (*Martes foina*) in der Oberlausitz. *Z. Säutetierkd* 64(Suppl. 5).

Berny, P. J., Buronfosse, T., Buronfosse, F., Lamarque, F., and Lorgue, G. (1997). Field evidence of secondary poisoning of foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) by bromadiolone, a 4-year survey. *Chemosphere* 35, 1817–1829. doi: 10.1016/S0045-6535(97)00242-7

Birks, J. D. S. (1998). Secondary rodenticide poisoning risk arising from winter farmyard use by the European polecat *Mustela putorius*. *Biol. Conserv.* 85, 233–240. doi: 10.1016/S0006-3207(97)00175-4

Brakes, C. R., and Smith, R. H. (2005). Exposure of non-target small mammals to rodenticides: short-term effects, recovery and implications for secondary poisoning. *J. Appl. Ecol.* 42, 118–128. doi: 10.1111/j.1365-2664.2005.00997.x

Christensen, T. K., Lassen, P., and Elmeros, M. (2012). High exposure rates of anticoagulant rodenticides in predatory bird species in intensively Managed Landscapes in Denmark. *Arch. Environ. Contam. Toxicol.* 63, 437–444. doi: 10.1007/s00244-012-9771-6

Clevenger, A. (1994). "Feeding ecology of Eurasian pine martens and stone martens in Europe," in *Martens, Sables and Fishers. Biology and Conservation*, eds S. W. Buskirk, A. S. Harestad, M. G. Raphael, and R. A. Powell (New York, NY: Cornell University Press), 326–340.

Cox, P., and Smith, R. (1992). "Rodenticide ecotoxicology: pre-lethal effects of anticoagulants on rat behavior," in *Proceedings of the Fifteenth Vertebrate Pest Conference* (Davis, CA: University of California).

Dowding, C. V., Shore, R. F., Worgan, A., Baker, P. J., and Harris, S. (2010). Accumulation of anticoagulant rodenticides in a non-target insectivore, the European hedgehog (*Erinaceus europaeus*). *Environ. Pollut.* 158, 161–166. doi: 10.1016/j.envpol.2009.07.017

Eason, C. T., Murphy, E. C., Wright, G. R., and Spurr, E. B. (2002). Assessment of risks of brodifacoum to non-target birds and mammals in New Zealand. *Ecotoxicology* 11, 35–48. doi: 10.1023/A:1013793029831

Eason, C. T., Milne, L., Potts, M., Morriss, G., Wright, G. R. G., and Sutherland, O. R. W. (1999). Secondary and tertiary poisoning risks associated with brodifacoum. *N. Z. J. Ecol.* 23, 219–224.

Elmeros, M. (2006). Food habits of stoats Mustela erminea and weasels Mustela nivalis in Denmark. *Acta Theriol.* 51, 179–186. doi: 10.1007/BF03192669

Elmeros, M., Topping, C. J., Christensen, T. K., Lassen, P., and Bossi, R. (2015). Spredning af anti-koagulante rodenticider med mus og eksponeringsrisiko for rovdyr. *Bekæmpelsesmiddelforskning* 159, 67.

Elmeros, M., Christensen, T. K., and Lassen, P. (2011). Concentrations of anticoagulant rodenticides in stoats Mustela erminea and weasels Mustela nivalis from Denmark. *Sci. Total Environ.* 409, 2373–2378. doi: 10.1016/j.scitotenv.2011.03.006

Erickson, W., and Urban, D. (2004). *Potential Risks of Nine Rodenticides to Birds and Non-target Mammals: A Comparative Approach,* Washington, DC: U.S. Environmental Protection Agency report. EPA Office of Pesticide Programs.

Evans, J., and Ward, J. L. (1967). Secondary poisoning associated with anticoagulant-treated nutria. *J. Am. Vet. Med. Assoc.* 151, 856–861.

Geduhn, A., Esther, A., Schenke, D., Gabriel, D., and Jacob, J. (2016). Prey composition modulates exposure risk to anticoagulant rodenticides in a sentinel predator, the barn owl. *Sci. Total Environ.* 544, 150–157. doi: 10.1016/j.scitotenv.2015.11.117

Geduhn, A., Esther, A., Schenke, D., Mattes, H., and Jacob, J. (2014). Spatial and temporal exposure patterns in non-target small mammals during brodifacoum rat control. *Sci. Total Environ.* 496, 328–338. doi: 10.1016/j.scitotenv.2014.07.049

Giraudoux, P., Tremollières, C., Barbier, B., Defaut, R., Rieffel, D., Berny, N., et al. (2006). Persistence of bromadiolone anticoagulant rodenticide in *Arvicola terrestris* populations after field control. *Environ. Res.* 102, 291–298. doi: 10.1016/j.envres.2006.02.008

Grolleau, G., Lorgue, G., and Nahas, K. (1989). Toxicité secondaire, en laboratoire, d'un rodenticide anticoagulant (bromadiolone) pour des prédateurs de rongeurs champêtres: buse variable (*Buteo buteo*) et hermine (*Mustela erminea*). *EPPO Bull.* 19, 633–648. doi: 10.1111/j.1365-2338.1989.tb01153.x

Jensen, T. S., and Nielsen, O. F. (1986). Rodents as seed dispersers in a heath—oak wood succession. *Oecologia* 70, 214–221. doi: 10.1007/BF00379242

Laakso, S., Suomalainen, K., and Koivisto, S. (2010). *Literature Review on Residues of Anticoagulant Rodenticides in Non-Target Animals Temanord.* Copenhagen, Nordic Council of Ministers. 541.

Lambert, M. S., Quy, R. J., Smith, R. H., and Cowan, D. P. (2008). The effect of habitat management on home-range size and survival of rural Norway rat populations. *J. Appl. Ecol.* 45, 1753–1761. doi: 10.1111/j.1365-2664.2008.01543.x

Lodal, J. (2010). *Resistens hos Brune Rotter: Monitering af Resistens hos den Brune Rotte i Danmark 2001–2008.* Miljøprojekt 1312, Danish Environmental Protection Agency / Miljøstyrelsen.

Lode, T. (1997). Trophic status and feeding habits of the European Polecat *Mustela putorius* L. (1758). *Mamm. Rev.* 27, 177–184. doi: 10.1111/j.1365-2907.1997.tb00447.x

McKenzie, T. L., Bird, L. R., and Roberts, W. A. (2005). The effects of cache modification on food caching and retrieval behavior by rats. *Learn. Motiv.* 36, 260–278. doi: 10.1016/j.lmot.2005.02.011

Murphy, E. C., Clapperton, B. K., Bradfield, P. M. F., and Speed, H. J. (1998). Brodifacoum residues in target and non-target animals following large-scale poison operations in New Zealand podocarp-hardwood forests. *N. Z. J. Zool.* 25, 307–314. doi: 10.1080/03014223.1998.9518160

Newton, I., Shore, R. F., Wyllie, I., Briks, J. D. S., and, Dale, L. (1999). "Empirical evidence of side-effects of rodenticides on some predatory birds and mammals," in *Advances in Vertebrate Pest Management,* eds D. P. Cowan and C. J. Frear (Fürth: Filander-Verlag), 347–367.

Nogeire, T. M., Lawler, J. J., Schumaker, N. H., Cypher, B. L., and Phillips, S. E. (2015). Land Use as a driver of patterns of rodenticide exposure in modeled kit fox populations. *PLoS ONE* 10:15. doi: 10.1371/journal.pone.0133351

Pelz, H. J. (2001). "Extensive distribution and high frequency of resistance to anticoagulant rodenticides in rat populations from Northwestern Germany," in

Advances in Vertebrate Pest Management, eds I. I. H. J. Pelz, D. P. Cowan and C. J. Feare (Fürth: Filander Verlag), 161–170.

Rattner, B. A., Lazarus, R. S., Elliott, J. E., Shore, R. F., and van den Brink, N. (2014). Adverse Outcome pathway and risks of anticoagulant rodenticides to predatory wildlife. *Environ. Sci. Technol.* 48, 8433–8445. doi: 10.1021/es501740n

Shore, R. F., Birks, J. D., Afsar, A., Wienburg, C. L., and Kitchener, A. C. (2003). Spatial and temporal analysis of second-generation anticoagulant rodenticide residues in polecats (*Mustela putorius*) from throughout their range in Britain, 1992-1999. *Environ. Pollut.* 122, 183–193. doi: 10.1016/S0269-7491(02)00297-X

Stradiotto, A., Cagnacci, F., Delahay, R., Tioli, S., Nieder, L., and Rizzoli, A. (2009). Spatial Organization of the Yellow-Necked Mouse: effects of density and resource availability. *J. Mammal.* 90, 704–714. doi: 10.1644/08-MAMM-A-120R1.1

Taylor, K. D. (1978). Range of movement and activity of common rats (*Rattus-Norvegicus*) on agricultural land. *J. Appl. Ecol.* 15, 663–677. doi: 10.2307/2402767

Taylor, K., and Quy, R. (1978). Long distance movements of a common rat (*Rattus norvegicus*) revealed by radio-tracking. *Mammalia* 42, 63–72. doi: 10.1515/mamm.1978.42.1.63

Topping, C. J., Dalby, L., and Skov, F. (2016). Landscape structure and management alter the outcome of a pesticide ERA: evaluating impacts of endocrine disruption using the ALMaSS European Brown Hare model. *Sci. Total Environ.* 541, 1477–1488. doi: 10.1016/j.scitotenv.2015.10.042

Topping, C. J., Hansen, T. S., Jensen, T. S., Jepsen, J. U., Nikolajsen, F., and Odderskaer, P. (2003). ALMaSS, an agent-based model for animals in temperate European landscapes. *Ecol. Modell.* 167, 65–82. doi: 10.1016/S0304-3800(03)00173-X

Vandenbroucke, V., Bousquet-Melou, A., De Backer, P., and, Croubels, S. (2008). Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. *J. Vet. Pharmacol. Ther.* 31, 437–445. doi: 10.1111/j.1365-2885.2008.00979.x

Vein, J., Grandemange, A., Cosson, J. F., Benoit, E., and Berny, P. J. (2011). Are water vole resistant to anticoagulant rodenticides following field treatments? *Ecotoxicology* 20, 1432–1441. doi: 10.1007/s10646-011-0700-7

Walker, L. A., Llewellyn, N. R., Pereira, M. G., Potter, E. D., Sainsbury, A. W., and Shore, R. F. (2010). *Anticoagulant Rodenticides in Predatory Birds 2009: A Predatory Bird Monitoring Scheme (Pbms) Report*. Lancaster: Centre for Ecology & Hydrology.

WHO (1995). *Environmental Health Criteria 175: Anticoagulant Rodenticides*. Geneva: World Health Organization.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Topping and Elmeros. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

REVIEW
published: 15 May 2017
doi: 10.3389/fenvs.2017.00020

Effects of Organic Pesticides on Enchytraeids (Oligochaeta) in Agroecosystems: Laboratory and Higher-Tier Tests

Jörg Römbke[1]*, *Rüdiger M. Schmelz*[1,2] and *Céline Pélosi*[3]

[1] ECT Oekotoxikologie GmbH, Flörsheim, Germany, [2] Grupo de Investigación en Biología Evolutiva, Facultad de Ciencias, Centro de Investigaciones Cientificas Avanzadas (CICA), Universidad de A Coruña, A Coruña, Spain, [3] UMR ECOSYS, INRA, AgroParisTech, Université Paris-Saclay, Versailles, France

Enchytraeidae (Oligochaeta, Annelida) are often considered to be typical forestliving organisms, but they are regularly found in agroecosystems of the temperate regions of the world. Although less known than their larger relatives, the earthworms, these saprophagous organisms play similar roles in agricultural soils (but at a smaller scale), e.g., influencing soil structure and organic matter dynamics via microbial communities, and having a central place in soil food webs. Their diversity is rarely studied or often underestimated due to difficulties in distinguishing the species. New genetic techniques reveal that even in anthropogenically highly influenced soils, more than 10 species per site can be found. Because of their close contact with the soil pore water, a high ingestion rate and a thin cuticle, they often react very sensitively to a broad range of pesticides. Firstly we provide a short overview of the diversity and abundance of enchytraeid communities in agroecosystems. Afterwards, we explore the available data on enchytraeid sensitivity toward pesticides at different levels of biological organization, focusing on pesticides used in (mainly) European agroecosystems. Starting with non-standardized studies on the effects of pesticides on the sub-individual level, we compile the results of standard laboratory tests performed following OECD and ISO guidelines as well as those of higher-tier studies (i.e., semi-field and field tests). The number of comparable test data is still limited, because tests with enchytraeids are not a regulatory requirement in the European Union. While focusing on the effects of pesticides, attention is also given to their interactions with environmental stressors (e.g., climate change). In conclusion, we recommend to increase the use of enchytraeids in pesticide risk assessment because of their diversity and functional importance as well as their increasingly simplified use in (mostly standardized) tests at all levels of biological organization.

Keywords: potworms, Annelida, Clitellata, plant protection products, laboratory, field, ecotoxicology

OPEN ACCESS

Edited by:
Johann G. Zaller, University of Natural Resources and Life Sciences, Vienna, Austria

Reviewed by:
Bruno B. Castro, University of Minho, Portugal
Bruno Silva Nunes, Center of Studies of the Environment and the Sea (CESAM), University of Aveiro, Portugal

***Correspondence:**
Jörg Römbke
j-roembke@ect.de

Specialty section:
This article was submitted to Agroecology and Land Use Systems, a section of the journal Frontiers in Environmental Science

Received: *17 February 2017*
Accepted: *24 April 2017*
Published: *15 May 2017*

Citation:
Römbke J, Schmelz RM and Pélosi C (2017) Effects of Organic Pesticides on Enchytraeids (Oligochaeta) in Agroecosystems: Laboratory and Higher-Tier Tests. Front. Environ. Sci. 5:20. doi: 10.3389/fenvs.2017.00020

INTRODUCTION

Plant protection products (PPP) or agricultural pesticides are commonly used in conventional agriculture world-wide, and their detrimental effects on non-target organisms are a major concern not only from a biodiversity perspective, but also considering the reduction of functions and services provided by soil ecosystems (Turbé et al., 2010; EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017).

Knowledge of pesticide effects on non-target organisms is therefore essential for sustainable agriculture. The role of soil fauna for maintaining ecosystem services has been studied intensively in the last decade (Mulder et al., 2011), with emphasis on the macrofauna such as earthworms (Brussaard, 2012). However, smaller-sized organisms such as enchytraeids also contribute to the functioning of agro-ecosystem (Didden, 1993). Enchytraeids occur worldwide in all soils with sufficient oxygen, moisture and nutrient supply, and they are regularly found even in intensively managed conventional agriculture (Pelosi and Römbke, 2016). The aim of this review is to gather all available information on pesticide effects on this important group of soil organisms.

Pesticides can harm non-target organisms either directly, by impacting their gene expression, behavior, reproduction, life cycle, or indirectly, by modifying interactions between individuals and populations (e.g., by affecting the prey of organisms but not the predators themselves). Laboratory single-species tests do not allow to address properly these complex effects of chemical exposure at the community level or higher, since they focus on single species under highly standardized conditions. So we have to understand what happens at the individual level but also to reveal the cascade of responses at the lower levels of biological organization and to adopt a more holistic assessment of higher hierarchical levels of ecological organization, i.e., populations, communities, ecosystems (European Commission, 2009). In order to manage ecosystem services successfully, we must understand how changes in community structure collectively affect the level and stability (resilience) of the ecosystem services over space and time (Kremen, 2005).

Enchytraeids or potworms (Enchytraeidae, Oligochaeta, Annelida) belong to the soil mesofauna (body diameter 0.1–2 mm). Today, about 206 species are listed in the key for terrestrial potworms of Europe (Schmelz and Collado, 2010) and 126 are known from Germany (Römbke et al., 2013). Probably 50 of them can be classified as common for Central Europe (Didden et al., 1997). The basis of enchytraeid taxonomy is the monograph of Nielsen and Christensen (1959, 1961, 1963) but since then, our knowledge has increased considerably with the description of new species (52 for Europe, Schmelz, personal comm.), enhanced morphological diagnoses (e.g., Rota and Healy, 1999), systematic revisions (e.g., Schmelz, 2003), and an updated guide to species identification (Schmelz and Collado, 2010). As in many other invertebrate groups it seems that a high cryptic diversity exists in the Enchytraeidae (Collado et al., 2012; Martinsson et al., 2015).

In this review we cover the effects of organic pesticides on enchytraeids in different land-use types and geographical regions. Each of these key terms is defined as follows: Pesticides are all chemicals being used against "harmful" organisms in agro-ecosystems, in particular herbicides, insecticides, and fungicides. We do not include copper, which is used as a fungicide, especially in vineyards. However, its fate and mode-of-action differs strongly from organic pesticides. Also effects of genetically modified plants/organisms are not addressed since this issue has already been covered in a review by Pelosi and Römbke (2016). Regarding land-use types, our review focuses on agro-ecosystems, i.e., crop sites, mainly on cereal crops, but also grasslands. Forests and urban sites were excluded, but information from such sites was sometimes included, in particular regarding methodological questions. All geographical regions with agricultural sites world-wide are included. However, with few exceptions, the majority of studies has been performed in the Continental and Atlantic biogeographic regions of Europe. Finally, all species of the family Enchytraeidae which occur in terrestrial habitats are covered. Taxonomic nomenclature follows Schmelz and Collado (2012), if not otherwise mentioned. It should be noted that we do not compare the sensitivity of enchytraeids toward pesticides with other soil invertebrate groups. Such comparisons are possible when looking at the results of standardized OECD (or ISO) tests, especially those with earthworms (*Eisenia fetida/andrei*), springtails (*Folsomia candida*) or predatory mites (*Hypoaspis aculeifer*). Such information can be found in Frampton et al. (2006) and Jänsch et al. (2006) for laboratory and field studies, respectively. Another more recent compilation has been made by Jarratt and Thompson (2009), who in particular compared the sensitivity of earthworms and enchytraeids toward pesticides.

To summarize, the aims of this review are (i) to give a short overview on the ecology, diversity and abundance of enchytraeid communities in agroecosystems, (ii) to compile ecotoxicological testing methods with enchytraeids, (iii) to list and discuss the effects of organic pesticides on enchytraeids at different levels of biological organization (including bioaccumulation). In this context, attention is also given to the interactions between other environmental stress factors (e.g., climate change) and pesticides. After summarizing these findings, the knowledge gaps regarding the use of enchytraeids in pesticide ecotoxicology will be pinpointed.

ENCHYTRAEID ECOLOGY

The ecology of terrestrial Enchytraeidae was firstly summarized by Didden (1993), but already in those days the main focus was on enchytraeids in forests. In such soils, potworms can occur in very high densities, up to several hundred thousand individuals per square meter (Peachey, 1963). Some species, e.g., *Cognettia sphagnetorum* in acid coniferous forests of Central and Northern Europe, play a key role in processes such as the decomposition of organic matter and nutrient cycling (Laakso and Setälä, 1999). In less acid soils, i.e., those in which earthworms do occur in higher numbers and biomass, enchytraeids are often less abundant and, thus, were considered of being less important (Petersen and Luxton, 1982). Since crop sites are usually kept within the neutral pH-range by fertilizing and liming, species with a preference for such soils are dominating, in particular those belonging to the genera *Fridericia* and *Enchytraeus*. According to Schärffenberg (1950) and Friberg et al. (2009), they are able to feed on plant-pathogenic nematodes and fungi at such sites.

In comparison to other land-use types the enchytraeid community of agricultural sites has rarely been studied (Pelosi and Römbke, 2016). Fortunately, in most cases standardized or at least very similar sampling methods were used (International

Organization for Standardization, 2007). Regular samplings have been made in The Netherlands (Schouten et al., 1999; Rutgers et al., 2008) and in Germany (Graefe, 1993; Römbke et al., 2000; Ruf et al., 2000; Graefe and Beylich, 2003). As a rough estimate and based on reviews of Petersen and Luxton (1982) and Römbke et al. (2002), the mean annual abundance at crop sites varies between 2,000 and 30,000 ind/m^{-2} with a biomass ranging from 110 to 640 mg dry weight (DW)/m^2. Depending on the soil properties, especially the pH value, these numbers can be 2–4 times higher in grasslands.

In general, not much is known about the diversity (species number, community composition etc.) of enchytraeids in agricultural soils, since the first key of European species was only published in the Late Fifties (Nielsen and Christensen, 1959, 1961, 1963). This situation improved only recently (Schmelz and Collado, 2010). Enchytraeid communities at crop sites have been classified as "impoverished" grassland communities (Jänsch et al., 2005). Römbke et al. (2013), after reviewing their occurrence all over Germany, evidenced the similarity of enchytraeid communities at arable and grassland sites. Recently, Pelosi and Römbke (2016) supported this view when reviewing the suitability of enchytraeids as indicators for agricultural management practices. **Table 1** exemplifies a "typical" enchytraeid community at crop sites in Germany, listing percentages of species occurrence and comparing them with those in grassland and deciduous as well as coniferous forests. Both the dissimilarity of the two forest sites vs. the two openland sites as well as the similarity of crop and grassland sites are evident. Notable differences between the latter two are lower densities of litter dwellers (e.g., *Buchholzia appendiculata*) and higher densities of very small species (e.g., *Enchytronia* sp.) and r-strategists (*Enchytraeus buchholzi*) at crop sites.

According to Graefe and Schmelz (1999), enchytraeid species differ with respect to their preferred occurrence in the soil profile. For convenience, these preferences can be combined into three groups as follows:

LD: litter dwellers (e.g., *Buchholzia appendiculata*, *Cognettia sphagnetorum*);
SD: soil dwellers (e.g., *Marionina clavata*, *Fridericia bulboides*, *Fridericia galba*);
IS: intermediate species (e.g., *Henlea perpusilla*, *Enchytraeus christenseni*).

Litter dwellers have sigmoid chaetae, they often move rapidly and with strong body contractions, and asexual reproduction by fragmentation is common. Litter dwellers usually feed on slightly to strongly decomposed remains of plants and on micro-organisms (bacteria and fungi), 80% of their diet being regarded to consist of micro-organisms and 20% of dead organic matter (Standen, 1973; Didden, 1993).

Soil dwellers are usually found in the uppermost 10 cm of the mineral soil, but exceptional depths down to 60–145 cm have been recorded (Dózsa-Farkas, 1991). Chaetae are straight distally, and body movements are often slower than those of litter dwellers. Some species are small, stress-tolerant worms (e.g., some *Enchytraeus* sp.), while *Fridericia*-species live in slightly acid to basic soils and vary considerably in size (Schmelz, 2003). Large species of this genus have strong body musculature, used for burrowing. *Fridericia* is by far the richest terrestrial genus of the family; up to now only few differences in habitat preferences have been found among the individual species. Most soil dwellers reproduce sexually. The diet is less well-known than in litter-dwellers, but seems to consist also of micro-organisms and dead organic matter (Schmidt et al., 2004).

Intermediate species occur in mineral soil and the organic layer. They form a heterogeneous group consisting mainly of r-strategists that often live close to the soil surface, independently whether there is a litter layer or not. Many intermediate species have short generation cycles, because of asexual reproduction, including fragmentation. Especially species of the genus *Enchytraeus* are well-known as indicators of stress, e.g., at grassland sites close to roads (Jänsch et al., 2005; Schlaghamerský, 2015). Not much is known about their feeding preferences, but many species of the genus *Enchytraeus*, for example, *E. albidus* or *E. crypticus*, can be bred in the laboratory for a long time on rolled oats.

Since the mid-fifties of the last century it is known that enchytraeids are found in clusters, meaning that there are considerable differences in their horizontal distribution on a small scale (Nielsen, 1954; Peachey, 1963). According to Didden (1993), they are occurring in more or less randomly distributed multispecies clusters of 100–1,000 cm^2 at arable sites. These differences may depend either on the heterogeneous distribution of resources (e.g., food) or on soil parameters (Chalupský and Lepš, 1985). However, Schrader et al. (2005) did not find a positive correlation between soil properties (e.g., sand content, amount of carbon of soil moisture) and the distribution of enchytraeids at German crop sites. Clusters may depend on reproduction activities, such as concurrent hatching from cocoons deposited in clusters (Nielsen, 1954).

The vertical distribution of enchytraeids at crop sites is strongly influenced by plowing, since organic matter is transported to deeper layers (Didden et al., 1997). As a result, the usual distribution found at sites without plowing—high densities close to the surface with decreasing numbers in deeper layers—can be changed to a more or less homogeneous density of enchytraeids within the plowing layer, but only as long as food is available there. Vertical migration of potworms is also caused by climatic factors (temperature, moisture) (Lagerlöf et al., 1989) and anthropogenic stress, such as pesticides applied to the soil surface (Römbke and Federschmidt, 1995).

Climatic factors, mediated by soil moisture and soil properties (e.g., pH) dominate the occurrence and activities of enchytraeids (Graefe and Schmelz, 1999; Maraldo and Holmstrup, 2010). In Central Europe, their population dynamics usually follow a seasonal pattern determined by temperature and precipitation: maxima occur in spring and autumn, while minima are observed in summer (caused by low moisture levels in soil) and in winter (because of low soil temperatures, especially when a snow cover is missing) (Nielsen, 1955; Didden, 1993). At grasslands and crop sites this pattern is often modified due to management practices (Pelosi and Römbke, 2016).

TABLE 1 | Species number, species composition, and percentage of species occurrence of enchytraeids at four land-use forms/habitat types in Germany (1st hierarchal level of habitat classification, juveniles not included), from Römbke et al. (2013).

Species	Cro (n = 24)	Gra (n = 38)	Dec (n = 34)	Con (n = 18)	Chi^2-Test Bonf.-corr.
Achaeta aberrans	12.5	5.3	**52.9**	38.9	**
Achaeta abulba	8.3	5.3	23.5	**66.7**	***
Achaeta affinis	8.3	5.3	**64.7**	27.8	***
Achaeta bohemica	4.2	7.9	17.6	**55.6**	**
Achaeta camerani	0.0	0.0	**55.9**	**55.6**	***
Buchholzia appendiculata	16.7	**63.2**	29.4	33.3	–
Cognettia sphagnetorum	8.3	10.5	**94.1**	**100.0**	***
Enchytraeus buchholzi	**95.8**	44.7	**50.0**	0.0	***
Enchytraeus christenseni	**91.7**	**63.2**	29.4	38.9	**
Enchytraeus lactaeus	**50.0**	13.2	5.9	0.0	**
Enchytraeus norvegicus	29.2	34.2	**55.9**	**50.0**	–
Enchytronia minor	**50.0**	31.6	0.0	0.0	***
Fridericia bisetosa	25.0	**50.0**	11.8	16.7	–
Fridericia bulboides	**83.3**	**86.8**	2.9	22.2	***
Fridericia christeri	**70.8**	23.7	0.0	0.0	***
Fridericia galba	**62.5**	**55.3**	23.5	11.1	*
Fridericia paroniana	**62.5**	15.8	8.8	0.0	***
Fridericia ratzeli	8.3	**65.8**	14.7	5.6	***
Fridericia striata	0.0	0.0	**55.9**	16.7	***
Henlea perpusilla	**83.3**	**55.3**	2.9	5.6	***
Henlea ventriculosa	37.5	**71.1**	0.0	0.0	***
Marionina clavata	0.0	2.6	**73.5**	**83.3**	***
Mesenchytraeus glandulosus	0.0	0.0	**76.5**	**16.7**	***
Mesenchytraeus pelicensis	0.0	0.0	26.5	**55.6**	***
Oconnorella cambrensis	0.0	0.0	**76.5**	**72.2**	***
Mean Ind./m^2 ± SD	20,165 ± 14,561	13,834 ± 11,312	51,241 ± 30,677	52,087 ± 43,837	
Mean species no./site ± SD	13.7 ± 4.3	12.2 ± 5.2	12.4 ± 5.5	9.2 ± 3.9	

*Cro, Crop sites; Gra, Grassland sites; Dec, Deciduous forest sites; Con, Coniferous forest sites. Typical species (= those with a frequency of more than 50% of all sites): given in bold. Asterisks indicate a statistically significant influence of habitat type on species distribution at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.*

Enchytraeids can be very important for soil functions such as maintaining soil structure and porosity, especially when earthworms are not abundant. For example, Van Vliet et al. (1993) and Topoliantz et al. (2000) found that potworms increase porosity through their tunneling activity and their deposition of fecal pellets, thus preparing micro-sites of high fertility. This activity could also influence the distribution of plant roots in the uppermost centimeter of the soil. Didden (1990) concluded that *Enchytraeus buchholzi* contributes to soil structure in much the same way as earthworms—just on a smaller spatial scale. Enchytraeids also influence nutrient-cycling processes, as demonstrated by high mineralization rates measured in the fecal pellets of *Buchholzia appendiculata* (Marinissen and Didden, 1997). Thus, enchytraeids can be classified as "biological regulators," i.e., they regulate in particular the abundance and activity of microbes through their feeding (Turbé et al., 2010).

HISTORICAL BACKGROUND: ENCHYTRAEIDS IN PESTICIDE TESTING

Among the first regulatory requirements for the risk assessment of pesticides neither soil organisms in general nor enchytraeids in particular played an important role. For example, in the first European Union document describing tests to be performed for pesticides (Commission of the European Communities, 1991), only tests with microorganisms, plants and earthworms were listed. Already 20 years earlier, the huge taxonomic and ecological complexity of soil organism communities became more and more obvious, best visible in the outcome of long-term research projects on the influence of acid rain on forest soils (e.g., Abrahamsen and Thompson, 1979; Bengtsson and Rundgren, 1982; Standen, 1984; Chalupský, 1989). Both high numbers and central ecological roles supported the idea to use enchytraeids in standard ecotoxicological tests. Although, lack of taxonomical knowledge and breeding difficulties hampered the development of test methods, the number of enchytraeid studies on the effects of chemicals started to grow, based on the experience made in acid rain research, and reached a peak in the late nineties. Within a short period of time, laboratory tests were developed. Since then, several reproduction tests have been internationally standardized (American Society for Testing and Materials, 2004; International Organization for Standardization, 2004; Organisation for Economic Co-operation and Development, 2004; Associação Brasileira de Normas Técnicas, 2012). In

addition, a standard semi-field test (Schaeffer et al., 2010) has been proposed, but so far, no field test is available. However, the inclusion of enchytraeids in the earthworm field test has been proposed (International Organization for Standardization, 1999). In parallel, OECD allows the use of enchytraeid species (besides earthworms) in soil bioaccumulation tests (Organisation for Economic Co-operation and Development, 2010). Currently the Enchytraeid Reproduction Test (ERT) is listed as an alternative or addition to earthworm tests in several regulations (e.g., European Plant Protection Organisation, 2003; VICH, 2005; European Chemicals Agency, 2014; EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017).

The need for enchytraeid tests in pesticide may increase in the future, since environmental risk assessment is currently changing: now, the main theoretical approach regarding the evaluation of ecological functions of organisms is the Ecosystem Service Approach (Millennium Ecosystem Assessment, 2005). Organism groups can be classified regarding their specific functions. According to the European Food and Safety Authority (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017), discussing further requirements for the registration of pesticides in Europe, enchytraeid populations or functional groups play an important role regarding the following functions at in-field sites (i.e., areas which are directly impacted by pesticides): biodiversity, genetic resources, cultural services, soil structure, nutrient cycling and food-web support. In contrast, they are considered of minor importance for pest control and natural attenuation. For all off-field areas (i.e., areas adjacent to sprayed fields, which could be grassland, forest etc.) all above-mentioned functions have to be protected.

MATERIALS AND METHODS

A literature review was carried out on the basis of keywords in ISI Web of Knowledge, using the "All Databases" option, with the following formula: "enchyt* or potworm* and pesticid* or herbicid* or fungicid* or molluscicid* or nematicid* or insecticid*" in Topics. In total, 2,741 publications were found. In a first step, publications were sorted and classified according to title, keywords and abstract. This selection revealed very different numbers for the specific parts of the review. For the individual and population levels, 226 papers were selected, while less than 15 publications were interesting for the assessment of pesticide effects on enchytraeids at the community level. In parallel, those authors which have been identified as relevant for our topic, were checked again. Afterwards, publication lists regarding *Enchytraeidae* in general (e.g., Schoch-Bösken and Römbke, 1993) or their use in ecotoxicology (e.g., Römbke, 2003) were checked. Special attention was given to the four reviews on their reaction to pesticides (i.e., Didden and Römbke, 2001; Frampton et al., 2006; Jänsch et al., 2006; Jarratt and Thompson, 2009). At the end, information from 302 papers was used for this review. About 5% of this list were not found via literature search but due to personal contacts (mainly older work or diploma reports).

The pesticides covered in this review are organic chemicals. We decided to exclude copper despite the fact that for more than 100 years it has been the active ingredient in a fungicide product, originally known as "Bordeaux Mixture." There is a huge amount of information available regarding the effects of copper on soil organisms in general (especially earthworms), but its effects on enchytraeids when sprayed as a pesticide are not yet covered. Copper is an essential element at low concentrations but toxic at high concentrations (Hopkin, 1989). Because of this complexity and the difficult distinction between effects of freshly sprayed copper fungicides and copper from other sources we decided that this topic requires a more detailed treatment than can be provided in our review. Sulfur and sulfur-based formulations were not explicitly looked for but they were not excluded either.

EFFECTS OF ORGANIC PESTICIDES AT DIFFERENT ORGANIZATION LEVELS

Response at the Sub-Individual Level

To our knowledge, the effects of pesticides on enchytraeids at sub-individual levels (i.e., molecular and cellular levels) have been addressed by only one Portuguese-Danish team. They investigated enchytraeid molecular and biochemical mechanisms in response to pesticide exposure using differential gene expression, as well as defense and cell injury biomarker activities (e.g., Howcroft et al., 2011; Novais et al., 2012c, 2014).

Recently, the Enchytraeid Reproduction Test (ERT) was modified in a way that embryotoxicity is covered (Gonçalves et al., 2015), measuring endpoints such as embryo development, number of embryonic structures, Calcium (Ca) channels quantification and hatching success in combination with macroscopic monitoring, histological and immunohistochemistry analysis. However, so far only data for cadmium are available. In parallel it has been checked whether changes in cellular energy allocation (CEA) could be used for the evaluation of the energetic status of an organism, but again not much experience is available so far (Gomes et al., 2015). In case this is possible, effects of chemical stressors could be determined more rapidly as in a full reproduction test.

Gene Expression

Based on a microarray (a tool that allows to detect the expression of thousands of genes at the same time) developed for *E. albidus,* Novais et al. (2012a) showed that the exposure to the organic pesticide phenmedipharm triggered a different set of genes in comparison to the exposure to the metal copper. As a consequence, the two groups of chemicals affected distinct biological functions. For instance, reproduction was only affected by pesticides, and lipid metabolic processes were only affected by metals. Moreover, three pesticides—the insecticide dimethoate, the herbicide atrazine and the fungicide carbendazim—affected biological processes in *E. albidus* in a dose-related manner, meaning that higher concentrations affected more transcripts than lower ones (Novais et al., 2012b). In this study, changes in gene expression, i.e., translation, regulation of the cell cycle and general response to stress, occurred after 2 days of exposure. Other studies showed that the transcriptional response was

time-dependent (Gomes et al., 2011). Transcriptional responses to the herbicide phenmedipham were higher after 2 days compared to 4 and 21 days (Novais et al., 2012c). After 21 days, no more biological responses to pesticide exposure could be detected, perhaps because of biological processes of regulation and stress management.

Most of the studies at the gene level have been carried out with *E. albidus*. Another microarray is available that allows the study of the expression of targeted genes in *E. crypticus* in response to a stressor (Ferreira et al., 2010; Castro-Ferreira et al., 2012). Microarrays are so far the only available tools to assess pesticide effects on enchytraeids at this level of organization. However, they have the disadavantage of targeting only certain genes present on the microarray, i.e., they do not allow to screen the genome without an a priori selection of the genes that are expressed. Moreover, microarrays are biased due to signal saturation (Zhao et al., 2014). To screen the genome expression without a priori selection and to quantify the level of expression of the differentially expressed genes, toxicogenomic approaches should be used, i.e., differential transcriptome analysis. However, so far these methods have not been standardized. It seems that there is a potential of using gene expression in risk assessment (Novais et al., 2012b), especially since a database containing genomic information for *E. albidus* is freely available (Novais et al., 2012a).

Biomarkers

Pesticide exposure produces oxidative stress through the generation of free radicals (i.e., reactive oxygen species, ROS) and lipid peroxidation induced in the tissues of mammals and other organisms (Banerjee et al., 2001). All organisms have defense systems including non enzymatic [e.g., vitamines) and enzymatic mechanisms [e.g., production of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase GST] that limit the potentially damaging effects of ROS on cells. Persistent detrimental changes in cell function occur only when all of the detoxification, repair and compensation systems are exceeded. Beyond this "threshold," cellular homeostasis is no longer ensured, and short and long-term, and often irreversible, negative consequences may occur (Mercurio, 2017).

Oxidative stress represents an imbalance between the production of ROS and the body defenses. Whereas direct measurement of ROS is difficult because of extremely short half-lives (Pryor, 1991), thiobarbituric acid reactive substances (TBARS) are more accessible. TBARS are degradation byproducts of fats formed during lipid peroxidation (Howcroft et al., 2009) that can be detected by the TBARS assay using thiobarbituric acid as a reagent. The measure of TBARS thus ensures the detection of a biochemical response even if some oxidative stress responses have been missed. Regarding cellular responses of enchytraeids to pesticides, several of these biomarkers have already been studied. Novais et al. (2014) assessed the effects of dimethoate, atrazine and carbendazim on the antioxidant defenses of *E. albidus* at different concentrations known to affect their reproduction (i.e., EC20, EC50, and EC90) and at different timings (i.e., 2, 4, 8, 14, and 21 days). They showed oxidative stress for all tested pesticides at sub-lethal concentrations. Moreover, atrazine induced damage in lipids, measured by lipid peroxidation. Once more, the time of exposure influenced the response of enchytraeids to pesticides since effects were more pronounced after 8 days of exposure than before (i.e., 2 and 4 days). Howcroft et al. (2009) found stronger effects on biomarkers after 3 weeks than after 2 days of exposure to Betanal (i.e., formulation with 157 g/L phenmedipham). This herbicide did not significantly alter the biomarker responses evaluated on *E. albidus* exposed during 2 days. However, the total glutathione and TBARSlevels increased, associated with an increase in activities of CAT, GPx, and GR and a decrease in GST activity after 3 weeks of exposure.

When neurotoxic pesticides are used, the transmission of the nervous influx can be disrupted (Howcroft et al., 2011). Cholinesterases (ChE) are a family of enzymes that catalyze the breakdown of some choline esters that act as neurotransmitters. It therefore plays a central role in the mechanism of neurotransmission. For instance, when acetylcholinesterase (AChE) activity is inhibited, the cholinergic receptors are overstimulated because of large amounts of acetylcholine accumulate in the synaptic junction. This can lead to behavioral changes and potentially to death (Howcroft et al., 2011). Similarly, gamma-aminobutyric acid (GABA) is the major inhibitory transmitter at neuromuscular synapses and synapses in the central nervous system, being also a biomarker of neurotoxic effects (Bicho et al., 2015). It has been shown that dimethoate caused ChE inhibition in *E. albidus*, indicating an impairment of the neuronal function, but further work is surely needed before such endpoints can be used in regulatory testing (Novais et al., 2014). Similarly, Howcroft et al. (2011) showed that a commercial formulation of phenmedipham inhibited ChE activity of *E. albidus* after 3 weeks of exposure, showing that ChE inhibition was a relevant biomarker for the studied pesticide.

Finally, along with the oxidative stress response, the energy reserves can provide information on the health status of the individuals. Because energy is a limiting factor for organisms, presence of pesticides can influence the trade-offs between energy allocated to stress management and life history traits, i.e., survival, growth, or reproduction. Organisms have to allocate their energy not only for maintenance, growth and reproduction but also for stress response (i.e., detoxification processes) while ensuring their basal metabolism and vital functions. Novais and Amorim (2013) studied the effects of three pesticides on cellular energy allocation (CEA) of *E. albidus* for up to 8 days, using concentrations analogous to the EC10, EC20, EC50, and EC90 values for these chemicals as previously determined in standard laboratory tests. A reduction in CEA was observed but only for atrazine at exposure times longer than 4 days. The authors explained that the low effects on CEA at concentrations known to affect reproduction (ECx) could indicate that the reduction in reproduction was not likely to be caused by a reduction in the total energy budget during the first 8 day of exposure. A complex endpoint such as CEA should thus always be complemented with measurements of the available energy reserves (Ea) and energy consumption (Ec).

Summary and Outlook

Despite the scarce literature, some advice can be given to assess the effects of pesticides on enchytraeids at sub-individual levels. Considering that the molecular and biochemical responses of *E. albidus* to pesticides appeared to be dose-related and time-dependent, it is recommended to test different concentrations of pesticides and different times of exposure. Experiment durations should be chosen to assess short, medium, and long-term responses, thus allowing to characterize gene expression and biomarker changes in a situation where reproduction occurred. Finally, this literature review makes evident a great need of further research at the sub-individual levels that addresses the effects of multiple stressors on the one hand and more ecologically relevant species of enchytraeids on the other. Moreover, although the reported references allow to infer the cascade of reactions that occurs when potworms are exposed to pesticides, few authors studied the link between different levels of biological organization (Novais et al., 2012b; Bicho et al., 2015) Finally, it is worth noting that to be able to discriminate between stress responses and natural ecological variations of biomarker expression, it is necessary to know the normal operating range (NOR) of a species. The first attempt was done by Novais and Amorim (2014) for *E. albidus* who provided a naturally varying ecological window for gene expression.

Within the last 10 years, interest in a mechanistic understanding on effects of chemicals in enchytraeids has increased (Spurgeon et al., 2008). Ecotoxicogenomic approaches can be used to analyze initial molecular and cellular effects, and the necessary sequence information is now available for enchytraeids, especially *E. albidus*. Therefore, it is now possible to address the biochemical basis of species sensitivity, the prevalence of multiple (and unexpected) modes of action, the consequences of chemical-induced change at the population and community level, and to derive a better understanding of the combined effects of pollutants (Spurgeon et al., 2008). While there is certainly an inherent (biological) variability, it seems that it will thus be possible to differentiate between the influence of test conditions and the effects of a stressor. In this context it is important to address metabolic effects of pesticides according to the outcome adverse pathway selection, by which already identified metabolic pathways of individual chemicals can be used as signal of chemical toxicity.

Response at the Individual and Population Levels

Test Methods

For about 50 years, enchytraeids have been used in laboratory studies with chemicals (e.g., Weuffen, 1968) and pesticides in particular (Way and Scopes, 1968; see also Römbke and Moser, 2002). These old data are difficult to evaluate because often agar or water was used as test substrate (e.g., Römbke and Knacker, 1989; Westheide et al., 1991; Christensen and Jensen, 1995; Kristufek and Ruzicka, 1995). Compared to tests performed with soil they are less useful for the assessment of chemicals since the exposure conditions are too artificial.

From the beginning, almost exclusively species of the genus *Enchytraeus* were tested, using both acute and chronic endpoints (Purschke et al., 1991) as well as bioaccumulation (Rüther and Greven, 1990). Criteria such as practicability (e.g., short generation times, easy identification, simple breeding) and sensitivity were used to find the most suitable species. For example, Brüggl (1994) compared the biology of *E. crypticus* and *E. minutus* (now *E. christenseni*) under laboratory conditions, measuring cocoon production, number of eggs per cocoon, and population growth. Originally *E. albidus* was the preferred test organism due to its size (ca. 2 cm), but *E. crypticus* became more popular due to its broader ecological range and higher practicality (e.g., shorter test duration, higher juvenile numbers, Kuperman et al., 2006). This recommendation has been confirmed several times (Castro-Ferreira et al., 2012; Voua Otomo et al., 2013). Bandow et al. (2013) proposed *Enchytraeus bigeminus* which reproduces asexually via fragmentation. Its handling and breeding is as easy as that of *E. crypticus*. In addition, several species from other genera have been proposed as test species, e.g., for forest litter the "typical" species of such habitats, *Cognettia sphagnetorum*—but testing this fragmenting species is difficult (Augustsson and Rundgren, 1998). Species of the genus *Fridericia*, being more relevant for agricultural soils, were also investigated in laboratory tests, e.g., in Korea (An and Yang, 2009) or China (Yang et al., 2012a).

For about 15 years, standardized chronic laboratory tests with enchytraeids have been available. The Enchytraeid Reproduction Test (ERT) (**Table 2**) was standardized in four versions, which differ only slightly: the ISO test (2004) covers retrospective sample testing from contaminated sites, the OECD test (2004) focuses on testing individual chemicals (particularly pesticides), the ASTM test (2004) has a broader approach and includes earthworms, and the ABNT test (2012) covers tropical conditions. Life duration (or the length of the full life-cycle) has been proposed as an additional endpoint more than 10 years ago (Pokarzhevskii et al., 2003). Recently Bicho et al. (2015) have demonstrated that this endpoint could be a worthwhile addition to the ERT.

Enchytraeids can avoid unfavorable environmental conditions, meaning that this behavior can be used as a quick effect endpoint: the organisms could choose between the control and a soil spiked with a pesticide. Such an enchytraeid avoidance test was developed by Achazi et al. (1999). Later on, the design of the standard earthworm avoidance test (International Organization for Standardization, 2008) was used as template, using small two-compartment test vessels. However, enchytraeids did not react more sensitively to the fungicides benomyl and carbendazim in these tests than in chronic tests (Amorim et al., 2005a). The experiments were repeated with different artificial soils (modified in terms of pH, clay or peat content) and different durations in order to improve the test methodology. However, sensitivity remained low and results were highly variable (Amorim et al., 2005b, 2008a,b). In addition, no clear relationship between avoidance behavior and ecologically more relevant endpoints such as reproduction could be established (Novais et al., 2010). When testing mixtures of pesticides with *E. albidus* Loureiro et al.

TABLE 2 | Overview of the properties of the Enchytraeid Reproduction Test (ERT), modified from Römbke and Moser (1999) and Römbke (2003).

Guideline + Reference	Guideline according to American Society for Testing and Materials (2004), International Organization for Standardization (2004), and Organisation for Economic Co-operation and Development (2004)
Test principle	Chronic, sub-lethal laboratory test
Test parameter	Mortality (adults), reproduction (number of juveniles)
Test duration	Range-Finding-Test: 2 weeks; main test: variable, depending on the species; *E. albidus*: 6 weeks; others species: 4 weeks
Test species	*Enchytraeus albidus* (Enchytraeidae) or *E. crypticus*; other species of this genus; in all cases originating from mass culture
Test substrate	Artificial soil: quartz sand, kaolin, peat, calcium carbonate and water (Organization for Economic Co-operation and Development, 1984); also field soils possible
Application of test substance	Mixed into the artificial soil; mixtures of contaminated and control soil also possible
Test conditions	10 adult (= clitellate) worms per test vessel (glass with lid; 0.2–0.25 L volume); temperature: 20 ± 2°C; permanently no light; moisture: 40–60% of the WHC_{max}., extraction of the juveniles using Bengal red; weekly feeding with rolled oats
Control	Untreated test substrate (e.g., artificial soil or a reference soil such as the German LUFA 2.2)
Validity criteria (control)	Mortality < 20% (adults); number of juveniles per test vessel (at the end of the test) > 25 (*E. albidus*) or > 50 (other species)
Test assessment	NOEC or ECx (treatment vs. control)
Reference substance	EC50 (reproduction) of Carbendazim: 1.2 ± 0.8 mg/kg
Limitations and remarks	Modifications: depending on the *Enchytraeus*-species and, for soil quality assessment, on the test soil (e.g., LUFA 2.2)

(2009) found antagonisms for dimethoate and atrazine, while synergisms were detected for lindane and dimethoate.

Some years ago, enchytraeids (especially *E. albidus*, *E. luxuriosus*) were included in the oligochaete standard bioaccumulation test (Organisation for Economic Co-operation and Development, 2010; **Table 3**). Regularly, this test is performed with earthworms, since effect data are usually available only for them. So far, few data from enchytraeid tests have been published (e.g., Bruns et al., 2001; de Amorim et al., 2002).

Effects of Insecticides

The effects of insecticides on enchytraeids are compiled in Table S1. In total, 12 active ingredients (plus one PPP metabolite) have been studied in 31 tests. The best known examples are dimethoate and lindane, which were investigated seven and five times, respectively. The former was used as a model chemical in the EU-SECOFASE-project (Løkke and van Gestel, 1998), while both were used in two Ph.D. theses (Amorim et al., 1999; Lock and Janssen, 2002). More than half (i.e., 16) of these tests were performed with *E. albidus*, 11 with *E. crypticus* (named *E. buchholzi s.l.* in three of them), three with *E. bigeminus* and one with *C. sphagnetorum*. Twelve tests were performed with OECD artificial soil, seven with the standard field soil LUFA 2.2 and the remaining 12 ones covered a wide geographical and pedological range of field collected soils (including one tropical soil). Fifteen tests were performed according to the OECD guideline No. 220 and four to the ISO guideline (including one draft version). Five avoidance tests were conducted according to an ISO draft guideline. Six tests were performed before the standard guidelines were fixed. With the exception of the avoidance tests, the performance of all tests did not differ much. LC50 values were determined in 17 tests and the $NOEC_{Reproduction}$ (alternatively, the EC10 is listed rarely) in 19 tests. Thirteen $EC50_{Reproduction}$ but only five $EC50_{Avoidance}$ values were found. When several endpoints were measured, mortality and (almost always) avoidance was found to be less sensitive than reproduction. Thus, usually the $NOEC_{Reproduction}$ was the most sensitive endpoint.

More contrasting results were found when looking at the effects of insecticides on enchytraeids in detail. We firstly discuss the tests in which only small effects were found [i.e., where the most sensitive endpoint is >10 mg a.i. (active ingredient)/kg soil DW (dry weight)], followed by those with effect values <10 mg a.i./kg soil DW—and in particular those, which have been tested several times, often in different soils. The value of 10 mg a.i./kg soil DW was chosen since even in worst case conditions, the exposure in the field will not exceed this concentration.

The first group consists of chlorantraniliprole (no effects on reproduction up to 1,000 mg a.i./kg soil DW, Lavtižar et al., 2016), chlorpyrifos (with just one $EC50_{Avoidance}$ value of 933 mg a.i./kg soil DW, Amorim et al., 2008b), toxaphene (no effects on survival and reproduction at 620 mg a.i./kg soil DW, Bezchlebová et al., 2007), parathion and its metabolite 4-nitrophenol (all reported effect values >20 mg a.i./kg soil DW, Römbke, 1991; Römbke and Moser, 1999). Natal-da-Luz et al. (2012) reported that spraying the insecticide diazinon on soil samples from Costa Rica did not cause adverse effects on *E. crypticus* ($NOEC_{Reproduction}$ of >16 mg ai./kg soil DW). Testing the effects of ethoprophos on E. *crypticus* in a Mediterranean soil resulted in an EC50 value of 68.5 mg a.i./kg soil DW (Leitão et al., 2014).

Alpha-cypermethrin belongs to the second group (i.e., effect values < 10 mg a.i./kg soil DW). Both NOEC and $EC50_{Reproduction}$ are below 5 mg a.i./kg dry soil (Hartnik et al., 2008). Malathion affects the reproduction of *E. albidus* at concentrations between 5 and 10 a.i./kg soil DW in two field soils and OECD soil, without any relation to their soil organic matter content (4.3 and 2.3 vs. 10%; Kuperman et al., 1999). This insecticide was more toxic to *E. albidus* juveniles than to adults in OECD soil.

In the following, some well-studied insecticides will be discussed. Puurtinen and Martikainen (1997) studied the effects of dimethoate on a small *Enchytraeus* species (probably *E. buchholzi s.l.*) in uncontaminated field soil at three different moisture levels (40, 55 and 70% of the soil WHC). It is less toxic

TABLE 3 | Overview of the properties of the Oligochaete Bioaccumulation Test (Organisation for Economic Co-operation and Development, 2010).

Guideline + Reference	Guideline according to Organisation for Economic Co-operation and Development (2010; see also American Society for Testing and Materials, 2004)
Test principle	Bioaccumulation test under laboratory conditions
Test parameter	Accumulation and elimination of chemicals
Test duration	14 days each for the accumulation and the elimination phase
Test species	*Enchytraeus albidus* or *E. luxuriosus*: *E. crypticus* also possible
Test substrate	Artificial soil: quartz sand, kaolin, peat, calcium carbonate and water (Organization for Economic Co-operation and Development, 1984); also field soils such as LUFA 2.2 possible
Application of test substance	Mixed into the test substrate; mixtures of contaminated and control soil also possible (use of radio-labeled substances highly recommended)
Test conditions	10 adult (= clitellate) worms (e.g., 5–10 mg wet weight per individual *E. albidus* and a length of about 1 cm) per test vessel (glass with lid; 10–20 g d.w. at a soil layer of 2–3 cm); temperature: 20 ± 2°C; permanently no light; moisture: 40–60% of the WHC_{max}., manual extraction of the worms
Control	Untreated test substrate (e.g., artificial soil or a reference soil such as the German LUFA 2.2)
Validity criteria (control)	Mortality < 20% (adults) of the total number of the introduced worms at the end of the test
Test assessment	Bioaccumulation factor: BAF or BSAF (lipid-normalized)
Limitations and remarks	Limited experience available so far (mainly from an international ring-test; Bruns et al., 2001)

in dry soil than in moist soil. Martikainen (1996) used the same study design, species and test chemical to investigate the effects of three soils with different texture. High soil organic matter content reduced the toxic effects. This insecticide showed low toxicity for *E. buchholzi s.l.* A similar result was found in two avoidance tests with *E. albidus*: the $EC50_{Avoidance}$ values were 58.3 mg a.i./kg soil DW (Amorim et al., 2008b) and 34 mg a.i./kg soil DW (Loureiro et al., 2009), respectively. Finally, low toxicity of dimethoate was also determined in a test with *C. sphagnetorum* (Løkke and van Gestel, 1998).

The insecticide lindane has been tested often, especially during the development of the ERT. Early work, e.g., that of Dormidontova (1973) could not be used since almost no information on test conditions or results is available. Loureiro et al. (2009) tested the toxicity of lindane on the mortality and avoidance behavior of *E. albidus* in LUFA 2.2 soil and found that the effects occurred in a similar range. Using OECD artificial soil, an LC50 of about 200 mg a.i./kg soil DW and a $NOEC_{Reproduction}$ of about 20 mg a.i./kg soil DW were found (Amorim et al., 1999). Depending on the soil type, Lock et al. (2002) found almost the same result ($EC50_{Avoidance}$ 172.5 mg a.i./kg soil DW) with *E. albidus* in OECD soil. Another example is the effect of lambda-cyhalothrin on the reproduction of the fragmenting species *E. bigeminus* which was examined under three different soil moisture levels (30, 50, and 70% of the soil WHC) (Bandow et al., 2013). A higher toxicity was observed in soil with lower moisture level. For lambda-cyhalothrin, the 21-day EC50Reproduction values at the three levels of soil moisture were calculated to be 1.33, 3.79, and 4.75 mg as/kg soil DW, respectively.

Finally, Chelinho et al. (2012) studied the effects of the insecticide/nematicide carbofuran on *E. crypticus* under tropical conditions in the laboratory, following basically the ISO standard (International Organization for Standardization, 2004). Actually, a new application method was used, intended to simulate pesticide spraying. The recommended dose of the fungicide carbofuran (1.178 mg/kg soil DW), twice the recommended dose, and a water control were sprayed on plastic trays (1.10 × 0.49 × 0.17 m length width × depth) containing a loamy soil. An EC50 value of 0.739 mg a.i./kg soil DW was determined for reproduction, i.e., lower than the predicted environmental concentration (PEC) after the recommended use of this pesticide. Chelinho et al. (2012) repeated the study with carbofuran and *E. crypticus* but this time they used soil which was applied in the field on plots varying in size between 3 × 1 and 4 × 2 m. About 18 h after spraying, the soil was collected for enchytraeid tests to be performed similarly as in the previous test. An EC50 value of 0.750 mg/kg soil DW was determined for the reproduction of *E. crypticus*, again a risk for potworms could not be excluded. In both tests no enchytraeid mortality was observed.

Effects of Fungicides

The effects of fungicides on enchytraeids are compiled in Table S2. Only six active ingredients have been studied in 32 tests so far. Twenty-two tests were performed with *E. albidus*, three with both *E. crypticus* and *E. bigeminus* as well as two with *E. coronatus*. *Fridericia ratzeli*—a species not cultured but collected from a grassland near Frankfurt—and *E. buchholzi* were used once. Fifteen and seven tests were conducted with OECD artificial soil or with the standard field soil LUFA 2.2, respectively. Two times LUFA 2.1 and 2.3 soils were used in the early days of the ERT development. Four field soils with varying properties and two forest soils (without and with pH modification (pH = 4.5 and 6.0, respectively) were used in order to evaluate the influence of soil acidity. Nineteen tests were performed according to the ISO guideline 16387, three avoidance tests were tested according to the respective ISO draft and three tests followed the OECD guideline. In seven tests performed during the ERT development no guideline was used. With the exception of the avoidance test, results of these tests were similar, as long as the same soil was used. LC50 values were determined in 17 tests and the $NOEC_{Reproduction}$ in eight tests. Nine $EC50_{Reproduction}$ and six $EC50_{Avoidance}$ values were found.

Azoxystrobin and chlorothalonil were tested just once. Pyrimethanil was studied in three tests, differing only in their soil moisture. The remaining tests were run with pentachlorophenol (PCP) and benomyl (both seven times) and carbendazim (14 times). In the laboratory tests listed in Table S2, PCP was not very toxic to *E. albidus* (the LC50 values are in a range of 15.5–444 mg a.i./kg soil DW). Mortality is clearly correlated with soil properties, especially with organic matter content, sand and pH.

The high toxicity of benomyl and in particular carbendazim to earthworms has been known for a long time (Stringer and Wright, 1973). Thus, it is used as reference substance in earthworm field tests (International Organization for Standardization, 1998, 1999) and was also selected as a model chemical during the development of the ERT (Römbke and Moser, 1999, 2002). Especially in the international ringtest, a huge data set was compiled (92 tests in total), allowing to assess the variability of this test system (Weyers et al., 2002). Variations were caused by a mixture of factors such as inherent biological variability or the level of experience of the participants. However, the ERT results were robust enough to standardize this method—a view which was supported by the first review on the effects of chemicals on enchytraeids (Didden and Römbke, 2001). Based on these experiences, carbendazim was selected as reference substance for the ERT (Römbke and Moser, 2002). One example from the ERT ringtest shows the effects of carbendazim on the reproduction of *E. albidus*, indicating that the numbers of juveniles per laboratory were close to the overall mean and that the EC10 values were almost always lower than the NOECs (see **Figure 1**; Römbke, 2003).

A clear difference in sensitivity between acute and chronic endpoints was found for carbendazim: the acute tests resulted in an LC50 of >10 mg/kg soil DW while the EC50 in the chronic test was 2.8–3.7 mg/kg soil DW. No significant differences were found between test runs following a NOEC design or those performed according to an ECx design, but the latter were less variable. The outcome of this ringtest formed the basis for the OECD, ISO, ASTM, and ABNT guidelines. The high chronic toxicity of carbendazim to enchytraeids has been confirmed several times (e.g., Castro-Ferreira et al., 2012). Avoidance behavior is not more sensitive than reproduction or even mortality (Amorim et al., 2005a). However, when spiking carbendazim in LUFA 2.2 soil and aging it for one, 14 or 28 days before starting the tests, Kobetičová et al. (2009) could show in an avoidance test that *E. albidus* clearly preferred the soil which was aged for 28 days, i.e., that one with the lowest availability of carbendazim.

Arrate et al. (2002) performed also the ERT with carbendazim, but they used *E. coronatus* instead of *E. albidus*—and tested the same compound in parallel in agar. Reduction in the number of juveniles was best explained by reduced hatching from cocoons, leading to a better understanding of the causes of toxic effects on these worms. In addition, the authors could show that the Mode-of-Action (MoA) of carbendazim significantly differs from those determined for other chemicals. Finally, it could be shown that the effects of this fungicide on *E. albidus* in laboratory tests differed in LUFA 2.2 soil in the absence or presence of 15‰ $NaCl_2$ (Silva et al., 2015).

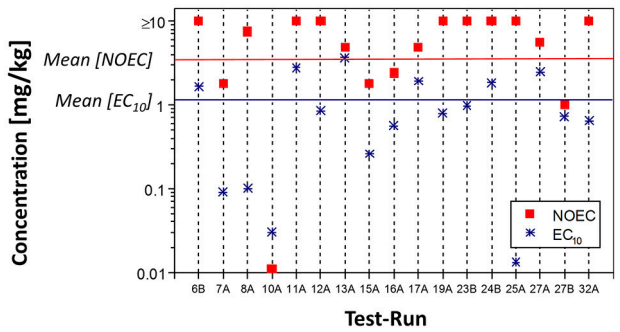


FIGURE 1 | Mean number of juveniles, presented as NOEC or EC10, of *E. albidus* in 17 different ringtest runs from 16 different laboratories, indicated here bei the codes on the x-axis (Römbke, 2003). Note that results were obtained in tests following either a NOEC design or ECx design (for details see Römbke and Moser, 2002).

Finally, Chelinho et al. (2014) investigated the effects of carbendazim on the reproduction and avoidance behavior of *E. crypticus* in five to eight Mediterranean soils plus OECD soil. The $EC50_{Reproduction}$ in the field soils differed between 0.73 and 1.27 (OECD: 0.89) mg a.i./kg soil DW, while in the avoidance tests less effects were determined [1.3–9.4 (OECD: 3.9) mg a.i./kg soil DW]. In both tests there was no difference between the effect size in OECD soil compared to the field soils.

Puurtinen and Martikainen (1997) studied the effects of benomyl on *Enchytraeus buchholzi s.l.* in uncontaminated field soil at three different moisture levels (40, 55, and 70% of the soil water holding capacity, WHC). The toxicity of benomyl decreased with increasing soil moisture content, but the mechanisms behind this behavior were not clearly understood. In a parallel study, Martikainen (1996) assumed that the high organic matter content of the soil reduced the toxic effects of this pesticide.

The fungicides azoxystrobin and chlorothalonil were tested in the ERT, using *E. crypticus*, but with a Mediterranean agricultural soil (Leitão et al., 2014). Effects were only observed at high concentrations (ca. 500–1,000 mg a.i./kg soil DW).

Finally, in enchytraeid reproduction tests with the fragmenting species *E. bigeminus,* pyrimethanil was examined under three different soil moisture levels (30, 50, and 70% of the soil WHC; Bandow et al., 2013). The highest toxicity was observed in soil with the lowest moisture level (i.e., EC50 of 435, 499, and 829 mg a.i./kg soil DW), probably due to synergistic effects of both the fungicide and moisture conditions.

Effects of Herbicides

The effects of herbicides on enchytraeids are compiled in Table S3. Only four active ingredients were tested in 23 tests so far. Bromoxynil was tested once, atrazine twice, and 2,4-5-T (banned already 20 years ago because of its carcinogenic properties) four times. All the other 16 tests were performed with phenmedipham, mainly as part of a Ph.D. thesis (Amorim et al., 2005a,b, 2008b). Out of these 23 tests, 17 were performed with *E. albidus*, five with *E. luxuriosus* and one with *Fridericia*

bulbosa (an invalid name according to Schmelz, 2003). The lack of tests with *E. crypticus* indicates that—with two exceptions—most of these tests were performed at least 10 years ago. OECD and LUFA 2.2 soils were used in five and eight tests, respectively. Eight tests were performed with, in total, three field soils. In the remaining test the very sandy LUFA standard soil 2.1 and the slightly humus-richer LUFA standard soil 2.3 were used. Eleven tests were performed according to the ISO guideline 16387, seven avoidance tests were tested according to the respective ISO draft and only one test followed the OECD guideline. In four tests no guideline was used, but they were conducted already in 1988/1989 (Römbke, 1989). LC50 values were determined in 14 tests and the $EC50_{Reproduction}$ values in 12 tests. Four $EC50_{Avoidance}$ values were found.

The low number of herbicides tests is probably caused by the fact that, due to their MoA, low effects on enchytraeids are expected. This expectation is fulfilled in the case of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) for which an extremely high LC50 value of 14,150 mg a.i./kg soil DW was found in OECD soil (Römbke, 1989). In the three LUFA soils, the LC50 values were by a factor of more than 10 lower. In contrast, atrazine affected the reproduction of *E. albidus* already at low concentrations (1–2 mg a.i./kg soil DW), independently from the endpoint (NOEC or EC50). Interestingly, avoidance behavior (EC50: 38 mg a.i./kg soil DW) was found to be an even less sensitive endpoint than mortality (LC50: 12 mg a.i./kg soil DW) (Novais et al., 2010).

Bromoxynil was found to cause quite high mortality of *Fridericia bulbosa*, even at low concentrations (Yang et al., 2012b). Thus, the authors recommended mortality as "valuable and sensitive" endpoint. This view is not supported by the many studies where reproduction and (sometimes) avoidance is more sensitive than mortality.

Phenmedipham is by far the best studied herbicide (16 tests), covering two species, three endpoints and five soils. Differences in sensitivity between species were low (i.e., within a factor of two in the same soils). There is an influence of soil properties on toxicity: LC50 values in OECD soil were by a factor of two higher than in LUFA 2.2. soil. Results from other field soils are somewhere in between, i.e., there is no clear difference to tests in OECD soil. Mortality and reproduction do not always show the same tendency: in tests with the field soil "Coi3" *E. luxuriosus* shows almost the same LC50 as in OECD soil, but the $EC50_{Reproduction}$ differs by almost a factor of 30 (*E. luxuriosus* reacts much stronger than *E. albidus*). Surprising differences were also found when performing avoidance tests in different soils but always with *E. albidus*: the $EC50_{Avoidance}$ varied between <1 and 252 mg a.i./kg soil DW. This difference might be caused by a combination of different soil properties but also by a lack of experience and/or the higher variability of the results of enchytraeid avoidance tests in general.

In one of the rare tests using soils from the Mediterranean region, Chelinho et al. (2014) investigated the effects of phenmedipham on *E. crypticus*, in 12 soils from Spain, Italy and Portugal plus OECD soil. Both the ERT as well as the enchytraeid avoidance test were used. The $EC50_{Reproduction}$ in the field soils differed between 3.8 and 32.8 mg a.i./kg soil DW. In contrast, the $EC50_{Avoidance}$ could only be determined in seven field soils, showing in general less toxicity (range: 19.1–>81 mg a.i./kg soil DW). Interestingly, the effects in OECD soil in the reproduction tests were almost always lower than in the field soils ($EC50_{Reproduction}$: 29.2 mg a.i./kg soil DW), while the opposite was determined in the avoidance tests with OECD soil: $EC50_{Avoidance}$: 14.1 mg a.i./kg soil DW). No significant relationships between soil properties and toxicity were found. Probably the range of properties of the selected field soils was too narrow to identify clearly their influence on toxicity.

Scoriza et al. (2015) studied the effects of the herbicide mesotrione which is used in forest restoration in Southern Brazil. Methodically, a combination of field experiments, focusing on soil arthropods, and laboratory tests with *E. crypticus* was used. Composite samples taken from the field before and one, eight and 22 days after application of 0.4. L/ha mesotrione were studied. Enchytraeid reproduction was severely affected in all samples after application. Thus, the authors recommend to use other herbicides (e.g., Fluazifop-P-butyl or Nicosulfuron), since these compounds did not affect enchytraeid reproduction. Since this herbicide was used in forests, it is not listed in Table S3.

Bioaccumulation of Pesticides in Enchytraeids

The best example for the use of enchytraeids (*E. albidus, E. luxuriosus*) in the standard OECD bioaccumulation test (Organisation for Economic Co-operation and Development, 2010) is a study performed with the insecticide lindane, which is, at least in Europe, no longer registered (Bruns et al., 2001; de Amorim et al., 2002). Lindane was quickly accumulated in both species in both soils, but was also quickly eliminated after transfer of the worms into clean soil (**Figure 2**). The experiment was repeated with both soils but only with *E. albidus* 1 and 2 months after spiking, i.e., the aging of this insecticide did reduce the bioaccumulation in the enchytraeids. Bioaccumulation factors differed between the two soils, probably because of the lower organic matter content in the latter, natural soil: the BAF was 12.1 in OECD soil and 22.0 (*E. albidus*)/36.1 (*E. luxuriosus*) in LUFA St. 2.2 soil. This difference in the bioaccumulation factors (BAF) of lindane in the two species is probably due to size-related differences and the respective volume: surface ratio (*E. albidus* is larger than *E. luxuriosus*; Amorim et al., 2002).

Summary

First of all, a standard test method (ERT) is available, which has been used in dozens of tests in various laboratories without any difficulties. The ERT allows some flexibility, i.e., various species of the genus *Enchytraeus* as well as different soils and endpoints can be used. The experiences with enchytraeid laboratory tests can be summarized as follows:

- Testing started with the large species *E. albidus*, but today it is used mainly when addressing other endpoints than those required in the standard tests. In "regular" standard tests, *E. crypticus* is used more often due to reasons of practicability. Differences in sensitivity could not be identified so far. Other *Enchytraeus* species (or species from other genera) cannot be

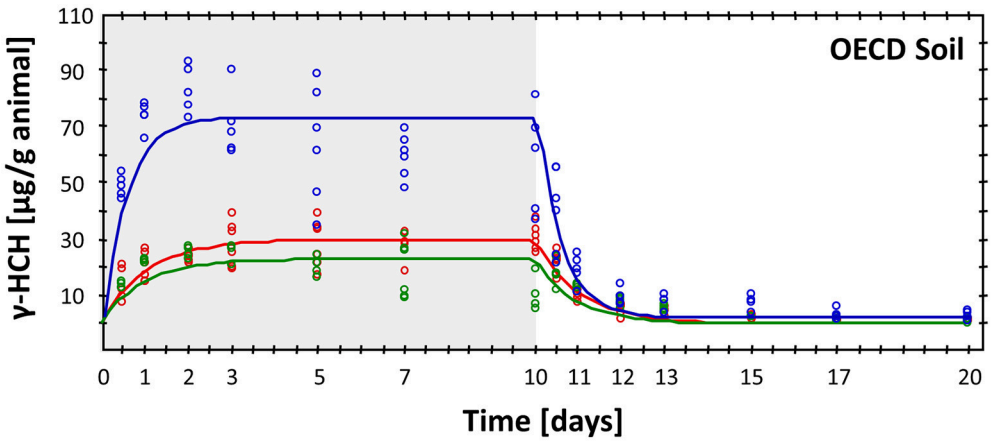


FIGURE 2 | Result of a bioaccumulation test of the insecticide lindane in *Enchytraeus crypticus* in OECD soil, showing both uptake (gray-shaded) and elimination phase (for details see de Amorim et al., 2002). Blue line, after freshly spiking; red line, 1 month after spiking and green line: two months after spiking.

recommended for the moment due to the low number of data available.
- When comparing endpoints, reproduction is by far the most robust and sensitive one. In contrast, the avoidance test is less useful, because of low sensitivity and high variability. Other endpoints scannot be judged due to low level of experience.
- Only few pesticides have been studied intensively, mainly the fungicide carbendazim, the insecticide dimethoate and the herbicide phenmedipharm. Most test data refer to pesticides which have been banned for years (e.g., PCP), while very few data are available for "modern" pesticides.
- The influence of soil properties on the effects of pesticides on enchytraeids is relatively well-studied, at least regarding texture and organic matter. Out of the 87 tests presented here, 31 tests were performed with OECD soil and 20 with LUFA 2.2 soil. The remaining 36 tests cover a wide range of soils from temperate regions—plus few examples from the Mediterranean and the tropics.
- An Enchytraeid Bioaccumulation Test has been standardized by the OECD some years ago, but despite its advantages in comparison to earthworms (shorter duration, smaller size and thus need of less space) it has rarely been used.

Response at Community Level

Methods Available

Various types of semi-field methods have been applied in the last 50 years to measure the effects of pesticides on enchytraeids, starting with microcosms. These are small vessels filled with field soil (rarely including plants), kept in the laboratory under controlled conditions. When all components of such a microcosm (including organisms) are selected by the experimenter, they are called gnotobiotic systems (gnotos (gr.) = known) (Mothes-Wagner et al., 1992; Born, 1993; Morgan and Knacker, 1994; Scott-Fordsmand et al., 2008; Schaeffer et al., 2010). The earliest known example is a study in Azalea cultures with various stressors, e.g., the herbicide DBCP (e.g., Heungens, 1968). Some early tests are known from Japan (Kitazawa and Kitazawa, 1980), which already studied combinations of (and thus interactions between) pesticides and ecological factors such as food addition. With few exceptions enchytraeid species were not identified. Most of the pesticides tested have been banned for a long time (e.g., pentachlorophenol, 2,4,5-T, aldicarb), while others such as carbendazim are still used today. Such microcosms are suitable for specific questions, e.g., the influence of pesticides on combinations of standard test soil invertebrates (*E. crypticus*, together with some springtail species and a predatory mite), but so far no standard guideline is available (Jensen and Scott-Fordsmand, 2012).

Gnotobiotic Approaches

Martikainen et al. (1998) were the first to study the effects of an insecticide (dimethoate) and a fungicide (benomyl), used alone or as mixture, in microcosms containing agricultural soil and indigenous soil fauna. They reported no effects on the total number of enchytraeids, but highlighted the added value of microcosm experiments in contrast to laboratory tests when studying complex questions.

The effects of carbendazim were also studied in a gnotobiotic microcosm, i.e., a plastic tube filled with sieved soil from the same site as the one used for the studies described below with Terrestrial Model Ecosystems (TME) (Burrows and Edwards, 2004). Enchytraeids (as well as other organisms) were added, exposed to the same concentrations of carbendazim and studied in the same way as in the TMEs. However, enchytraeids were not affected, meaning that this test system was—at least for this endpoint—not able to predict effects which were found both in the TMEs and in the field. A similar approach has later been used by Jensen and Scott-Fordsmand (2012) who designed a soil multi-species (SMS) test system consisting of one potworm species (*E. crypticus*), four springtail species and one predatory mite species. With an additional stress factor (here: the predatory mite), the springtails reacted much more sensitively to the insecticide ivermectin (primarily known as a veterinary pharmaceutical) than when exposed alone. Since the mites fed

selectively more on enchytraeids than on springtails it is likely but not yet proven that the same phemomenon could happen with potworms.

Sechi et al. (2014) used the same SMS to study the effects of the insecticide alpha-cypermethrin on the same artificial community as described above (including *E. crypticus*). A community EC50 of 1.26 mg/kg soil DW was determined—a value which is significantly lower as the EC50 value measured in a single-species test with the same enchytraeid (4.91 mg/kg soil DW) (Hartnik et al., 2008).

Terrestrial Model Ecosystems (TME)

The best-known example for a "real" semi-field method uses Terrestrial Model Ecosystems (TMEs) (Knacker et al., 2004; Förster et al., 2006; Moser and Römbke, 2007; Moser et al., 2007; Scholz-Starke et al., 2013; Bandow et al., 2016), which was originally called a "terrestrial soil-core microcosm test" (American Society for Testing and Materials, 1993). TMEs are non-disturbed soil cores (diameter 15–45 cm; height 30–60 cm), taken from the field and containing the original soil organism community except, to a certain degree, the macrofauna, especially those species living on or close to the soil surface. TME studies can be performed both in-house, e.g., in temperature–controlled rooms (**Figure 3A**) as well as out-doors (**Figure 3B**). A proposal for an OECD test guideline is available (Schaeffer et al., 2010).

In the first TME study with pesticides, Römbke et al. (1994) studied the effects of the insecticide parathion and the herbicide formulation Ustinex (consisting of two active ingredients, amitrole and diuron) on the enchytraeid community of a Central German grassland. Both pesticides were sprayed in two concentrations on top of the intact soil cores. Samples were taken 1 month before application and 1, 2, 3, and 4 months after application. Enchytraeid species number and total abundance were not negatively affected, except in the treatment with the higher parathion concentration. In fact, in the TMEs with the low herbicide concentration their numbers actually increased; maybe because the insecticide eliminated Collembola (food competitors) and predatory mites (main predator).

Similarly, Moser et al. (2007) used intact soil columns collected from three grasslands in Germany, Great Britain and The Netherlands and one arable site in Portugal. They applied six different concentrations of the fungicide carbendazim as formulation Derosal®. At all sites, the genus *Fridericia* was most negatively affected by the pesticide, mainly 8 and 16 weeks after the application, followed by species of the genus *Henlea*. Many *Achaeta* and *Enchytraeus* species did not decrease or even partly increased (**Figure 4A**). In general, enchytraeids were not affected by the two lower concentrations (in fact their number increased slightly above control level) but showed a strong decline in the TMEs treated with the two higher concentrations. During the testing period, no indication of recovery could be seen.

At the Flörsheim site, a different effect pattern was found (**Figure 4B**), meaning that the lowest concentration caused only small and not lasting effects. In the three higher concentrations, the effects were stronger until week eight after application. With the exception of the two highest concentrations, the control level was reached within the study duration. The differences between the enchytraeid effect patterns at the different test sites are probably mainly caused by differences in soil properties. In addition, the different species composition of the enchytraeid communities might have played a role.

The authors explained that these results could be attributed *"to the different ecological requirements [...] of the different genera."* For example, *Fridericia* and *Henlea* species are K-strategists (i.e., long life duration, slow reproduction) whereas *Enchytraeus* species are r-strategists (i.e., short life duration, rapid reproduction) (Graefe and Schmelz, 1999). This ecological difference may affect recovery of the different species as well. In any case, the low abundance of enchytraeids belonging to the genus *Fridericia* would indicate a risk of high application rates of carbendazim when using the EU requirements relevant at that time (Weyers et al., 2004). Interestingly, the effects of carbendazim on the total abundance of enchytraeids were correlated with those found when measuring organic matter decomposition (using the filter-paper method) but not with those on the feeding rate as measured in the bait-lamina test (Förster et al., 2004).

Scholz-Starke et al. (2013) found 17 enchytraeid species in 45 TMEs they had collected at a German meadow site. Enchytraeids were sampled after 1, 26, and 149 days after lindane applications. The authors found no significant effects of the pesticide at concentrations ranging from 0.032 to 3.2 mg/kg soil DW on total abundance or that of individual species. Finally, Bandow et al. (2016) found that the fungicide pyrimethanil did not affect the community composition (consisting of *Enchytraeus buchholzi, E. bulbosus, E. dichaetus, Fridericia bulboides, F. pretoriana, F. tuberosa,* and another *Fridericia* species) in a TME experiment performed in Portugal, but they reported negative effects from a similar experiment performed in Germany. In the latter one, the strongest effects were found in dry soil, particularly for *Fridericia connata* after 8 weeks of exposure. It is not clear whether different community composition or soil properties may have caused the different outcome.

Field Approach

In order to survey enchytraeids in the field, soil samples are taken with a corer (diameter usually between 5 and 7.5 cm). Theses samples are separately placed onto sieves hanging in plastic bowls filled with water, and the enchytraeids are driven via wet-extraction from the soil. This procedure has been internationally standardized (International Organization for Standardization, 2007). Species identification is only possible with living specimens, which limits the number of samples that can be handled in parallel. Species determination via genetic methods (DNA barcoding and meta-barcoding) is a promising alternative (Orgiazzi et al., 2015) since the establishment of DNA sequencing as a cheap routine laboratory procedure. However, there is still a problem with the interpretation of the results, because the number of reliable data sets combining genetic and morphological information is small.

The first field study covering the effects of pesticides on enchytraeids, among others, was performed in Northern Germany (Weber, 1953). Edwards et al. (1968) and Edwards and Lofty (1971) described effects of insecticides (i.e.,

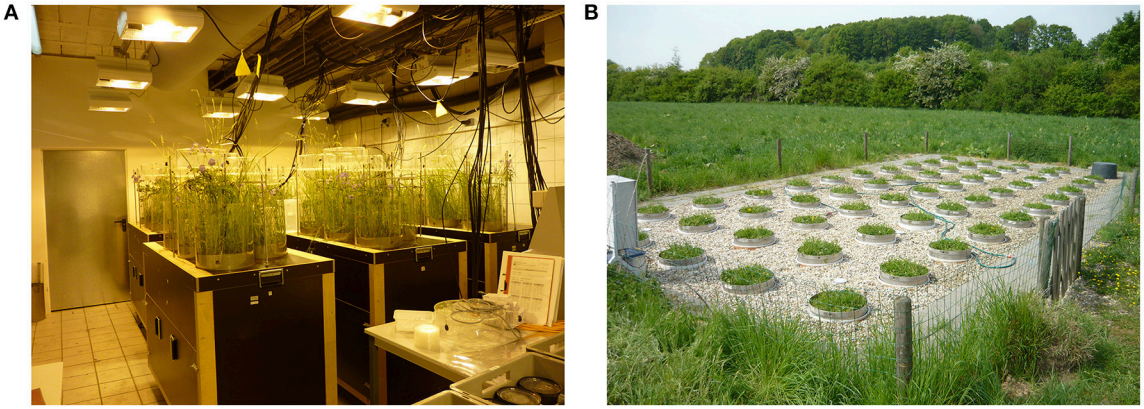


FIGURE 3 | (A) TME in-house facility (ECT GmbH) (B. Förster). **(B)** TME outdoor facility (RWTH Aachen/gaiac) (B. Scholz-Starke).

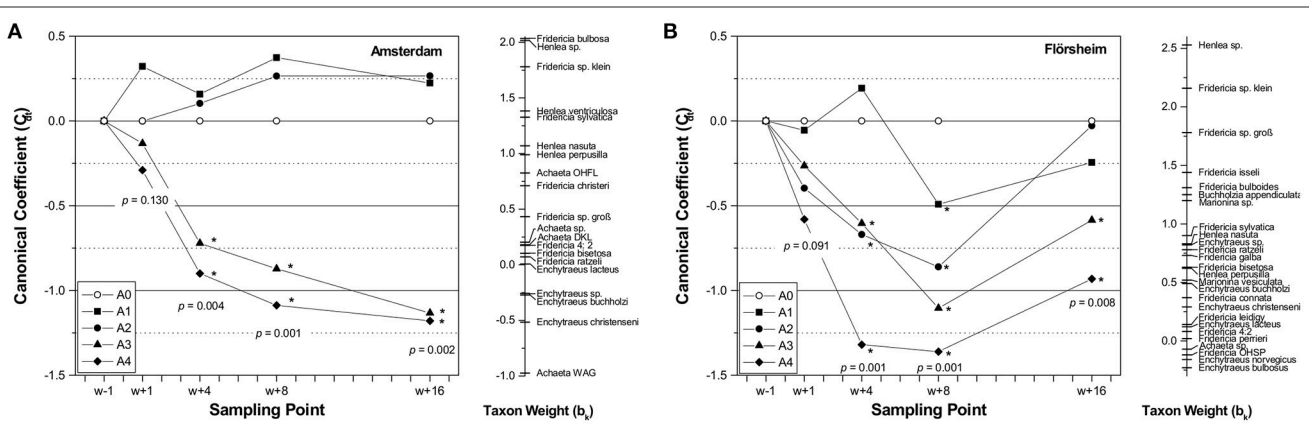


FIGURE 4 | (A) PRC (Principal Response Curve) for the effects of carbendazim on the Enchytraeid species community in the TME test of Amsterdam. Presented is the canonical coefficient (Cdt) of the different application rates at the sampling points w+1, w+4, w+8, und w+16 (weeks after application) and the taxon weight (bk) for all taxa. For each sampling point the *p*-value (Monte Carlo permutation test; 999 permutations) for the comparison of all application rates (including control) is given. Significant differences of the PCA sample scores compared to the control are indicated with an asterisk (Williams *t*-test; 2-sided, $p \leq 0.05$). Application rates: A0 = control, A1 = 0.36, A2 = 2.16, A3 = 12.96, A4 = 77.76 kg carbendazim/ha) For further details see Moser et al. (2007). **(B)** PRC (Principal Response Curve) for the effects of carbendazim on the Enchytraeid species community in the TME test of Flörsheim. For further explanations see the legend of **(A)**.

chlorfenvinphos) or nematicides (i.e., methomyl, dazomet or aldicarb) on enchytraeids at agricultural sites. Voronova (1968) studied the effects of the insecticide Sevin on enchytraeids in the Taiga region of Russia. Since hand-sorting was used as extraction method the results of these studies are not reliable. However, Van den Brande and Heungens (1969) used already wet extraction when studying the effects of the nematocide disinfectant DD on enchytraeids in plots with Begonia. However, the outcome is hardly useful since samples were not replicated. Martin (1975) found no effect of the pesticide fenithrothion on enchytraeid abundance in a New Zealand pasture at field-relevant concentrations. The same result was reported by McColl (1984) when studying benomyl (18.6 kg a.i./ha) and phenamiphos (18.6 kg a.i./ha) on grasslands in New Zealand. In contrast, Popovici et al. (1977) studied the effects of atrazine at two concentrations (5 and 8 kg/ha) on enchytraeids–among other soil organisms –, and observed a quick decrease in enchytraeid numbers at both concentrations 1 month after application. However, numbers increased 4 months after application at the lower concentration. In a study using small field plots, Römbke et al. (1994) applied an insecticide (parathion) or a herbicide mixture (Amitrole/Diuron) at the highest recommended application rate or a 5-fold higher concentration rate. A strong increase in enchytraeid abundance and biomass occurred at the lower herbicide rate, but the high rate caused a decrease of 50% for both endpoints. Both application rates of the insecticide did not affect enchytraeid abundance or biomass. However, so far no standardized field test method is available (e.g., Römbke et al., 2009).

Enchytraeids may also avoid chemicals by vertical migration, which has been observed in a field study in a German grassland (Römbke and Federschmidt, 1995). Carbendazim was sprayed on small plots at two concentrations and the abundance, biomass and diversity of the enchytraeid community was studied for two

years, divided into an application phase and a recovery phase. Negative reactions on the enchytraeid community were found at concentrations lower than those identified in the laboratory. However, since soil properties were not the same in the laboratory and field tests the results are difficult to compare.

The effects of the fungicide carbendazim (i.e., the formulation Derosal®) on enchytraeids were not only determined in TME tests (Knacker et al., 2004), but also in parallel in the field, using the same concentrations (Moser et al., 2004). This work was done by different partners at one arable site (Coimbra, Portugal) and three grassland sites: Amsterdam (The Netherlands), Bangor (Wales, England), and Flörsheim (Germany). No differences regarding enchytraeid total abundance or number of species were found between the respective TMEs and field sites in the controls. Effects of carbendazim were most pronounced when looking at the abundance of worms of the genus *Fridericia* (especially 8 and 16 weeks after application), while the abundance of the genera *Achaeta* and *Enchytraeus* was not affected. The observed effects did not differ between TME tests and the respective field validation studies (Weyers et al., 2004). Due to high variability of data in both tests, NOEC-values could often not be determined. The EC50-values (based on total abundance) derived from the TME tests and the field validation study indicate that the reproducibility (i.e., the variation between the partners) of the EC50-values was reasonable, although different soils were used at the different sites. The EC50-values, based on total abundance, ranged between 0.7 and 37.8 mg a.i./kg, which is very similar to those values based on the abundance of the most abundant genus *Fridericia* (i.e., 0.9–24.7 mg a.i./kg soil DW). On the contrary, the EC50-values based on the endpoint number of species was less sensitive (9.5–116.2 mg a.i./kg soil DW). Since no genus was consistently more sensitive than the other genera, it is recommended to include the species level in the assessment of field studies. As in the TME study performed by the same authors, effects on enchytraeids at the four field sites were not correlated with those found in the bait-lamina test but with those from organic matter decomposition tests (Förster et al., 2004).

Potworms have been recommended repeatedly for monitoring programs or assessment schemes, e.g., in the context of post-registration monitoring of pesticides (Schouten et al., 1999; Barth et al., 2000; Jänsch et al., 2005; Bispo et al., 2009). Proposals are available for reference values (diversity, species number, or abundance) of enchytraeids at different sites in the Netherlands and Germany (Rutgers et al., 2008; Beylich and Graefe, 2009).

Recovery

Recovery of enchytraeids in agro-ecosystems after pesticide exposure has not been studied so far (Kattwinkel et al., 2015). All available information is from forest sites. In a beech forest in Southern Germany, two model pesticides, the fungicide PCP and the herbicide 2,4,5-T were applied bimonthly on small field plots (25 m^2) for about 2 years (Römbke, 2001). This study aimed to understand recovery processes after strong stress. Since very high concentrations of these pesticides were used, the enchytraeid populations were strongly affected (especially in a year with a long period of drought) during the application period (Römbke, 1988). After stopping the applications of PCP, enchytraeid abundance started to recover less than half a year later in plots with the lower application rate, while abundance remained significantly lower at the higher application rate for about at least one more year. Thus, it could be shown that such recovery depends strongly on pesticide exposure (here given as applied amount) in interaction with general (mainly climatic) factors (**Figure 5**)—a scheme which probably is true also for agro-ecosystems. As a side effect, enchytraeid abundance increased to numbers even higher than in the controls, probably because during the application period the litter layer (i.e., food) was not degraded. This picture is mainly caused by r-strategists (i.e., those adapting quickly to changing environments) such as *Cognettia sphagnetorum*—a potworm which can reproduce by fragmentation (Nielsen and Christensen, 1959). The recovery pattern on the plots treated with 2,4,5-T was very similar.

Summary and Outlook

In 1999, Cortet et al. (1999) firstly summarized the experiences with PPP effects on enchytraeids, listing five papers. Two years later, Didden and Römbke (2001) summarized the information provided in about 30 papers and identified issues deserving further attention. With the improvement of extraction methods and a better availability of taxonomic keys in the past 10 years (Schmelz and Collado, 2010), our knowledge on enchytraeid taxonomy and ecology as well as their reaction to pesticides have certainly increased considerably, not only in Europe but also for example in Brazil (Chelinho et al., 2012; Assis, 2015). On the other side—and despite the fact that standardized methods are available and new ones are in the making (e.g., Bicho et al., 2015)—the use of enchytraeids in regulatory assessment schemes is still very limited. Indeed, enchytraeids played only a minor role in the risk assessment of pesticides in Europe during the past 25 years, mainly because there were no legal requirements for such tests. However, this situation is going to change, since in the "Draft Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms" (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017), Enchytraeidae are one out of seven organisms groups for which Specific Protection Goals (SPGs) are going to be defined, meaning that the relevance of these organisms (and the need to study them) is surely more acknowledged than it has been in the past.

Only few studies addressed the effects of pesticides on enchytraeid communities either under semi-field or field conditions so far. A draft standard method exists for a semi-field method, i.e., TMEs (Schaeffer et al., 2010), but nothing like that is available for field tests. However, the standard earthworm field study (International Organization for Standardization, 1999) could be improved by adding potworm abundance and diversity as additional endpoints. In any case, a central part of practical work (extraction of enchytraeids from soil samples) is already covered in an ISO guideline (International Organization for Standardization, 2007), focusing on monitoring enchytraeids. More difficult is the situation for gnotobiotic semi-field approaches, since several proposals have been made and the amount of information about their pros and cons is still limited. The most promising method is the SMS (a simplified food-web

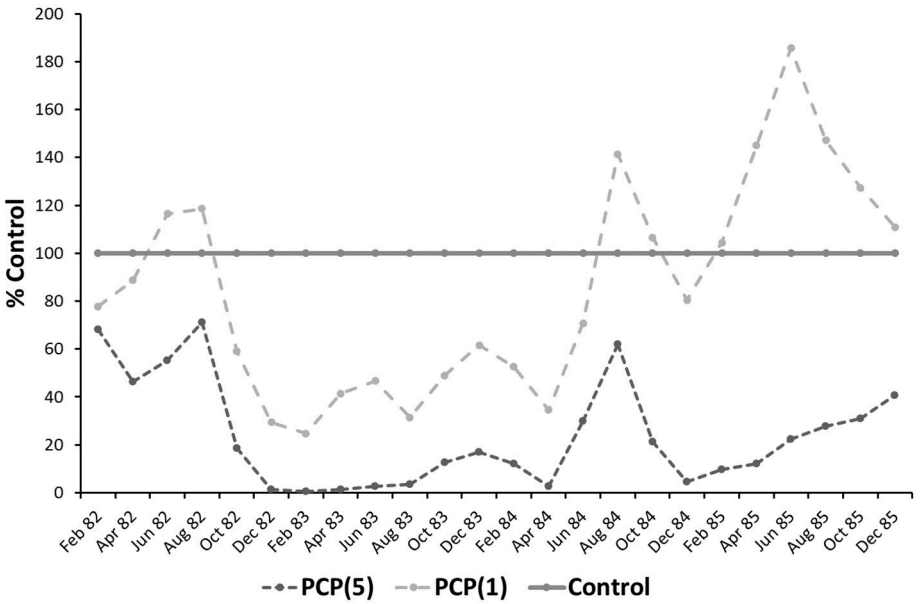


FIGURE 5 | Effects of two concentrations of the fungicide PCP (light gray dotted line: 0.5 kg/m^2; dark gray dotted line: 5 kg/m^2) on the enchytraeids of a beech wood forest (bimonthly application: 1982–1983; recovery time: 1984–1985) in percent of the control (solid darkline) (for details see Römbke, 2001).

approach, consisting of springtails, enchytraeids and predatory mites; e.g., Menezes-Oliveira et al., 2014). In fact first studies aiming at its standardization have been published (e.g., Sechi et al., 2014).

The experiences with enchytraeid higher-tier testing can be summarized as follows:

- In these tests total abundance was often the main (or only) endpoint. Higher sensitivity can be expected at the genus or species level, but there are difficulties due to the impracticability of enchytraeid taxonomy. However, this situation is going to improve due to better keys (at least for Europe) as well as the upcoming genetic methods.
- Regarding other endpoints, experience is limited. Total biomass and abundance seem to be correlated. In the few studies in which enchytraeid species were identified a quite high number was found even at crop sites, meaning that diversity is worthwhile to be included.
- The available information is not yet sufficient to clarify whether individual species or genera are more sensitive to a specific pesticide than others. However, species of the genus *Fridericia* (i.e., K strategists) are more affected by carbendazim than other enchytraeids, which may be due to different exposure or to physiological differences.
- The number of pesticides studied in higher-tier tests with enchytraeids is very low; besides some old compounds and only few currently used PPPs have been investigated. The one exception is carbendazim, which has already been used in a European ring test, both in TMEs and the field. Sensitivity of earthworms and enchytraeids differed by about the factor of three in this ring-test, meaning that each organism groups would have indicated a risk.
- Despite its small number, the results of semi-field and field studies can provide relevant information for the environmental risk assessment of pesticides, as shown for carbendazim. Effects of herbicides may be less relevant in terms of direct toxicity but could be considered as an example of indirect effects (e.g., via change in soil moisture due to plant removal).
- By definition, higher-tier studies do include site-specific properties (which means mainly soil properties but also climatic factors) and this inclusion of realism has to be addressed in current risk assessment procedures. In fact, in modern TME facilities realistic climatic scenarios can be simulated. In this context the "Normal Operating Ranges" (NORs) of at least the most common enchytraeid species should be determined, preferably using soil biodiversity data bases (e.g., Burkhardt et al., 2014).
- The information available from higher-tier studies does allow to draw important conclusions, for example when addressing the question how much the composition of invertebrate communities determines the level of ecotoxicity (Sechi et al., 2014). Another example is the interaction between (changing) climate parameters with chemicals, which in turn can cause unpredictable changes in community structures (Menezes-Oliveira et al., 2014).
- Last but not least such semi-field and field studies are necessary to "validate" the results of lower tier studies and/or for the development of realistic modeling approaches.

KNOWLEDGE GAPS AND PERSPECTIVES

As shown above there are still various issues regarding the effects of pesticides on enchytraeids which need further research. Among them are basic questions referring to their biology and ecology, such as:

- How much differs sensitivity among individual species? Are the species used in standard tests (mainly *E. albidus*, *E. crypticus*) representative in terms of sensitivity for the whole family?
- It is possible to use metabolic pathways of enchytraeids in order to address metabolic effects of pesticides and to reveal the cascade of responses at the lower levels of biological organization?
- How do enchytraeids incorporate pesticides, i.e., which are the main exposure pathways and how much do they depend on soil properties or on chemical characteristics (Gomes et al., 2011; Peijnenburg et al., 2012)?
- Which endpoints are the most sensitive, relevant, robust and practical ones? Reproduction surely comes first but is time-consuming. Could genetic markers take over this role while being quicker to perform (Amorim et al., 2011)?
- What about the trait concept–can we identify morphological/ecological groups which may be more relevant than individual species?
- Is it possible to overcome taxonomic burdens by building up a database containing enchytraeid gene sequences so that enchytraeid diversity could be an "easy" endpoint in field studies or monitoring approaches?

In a regulatory context, further questions have to be answered (most of them are important for other soil organisms too; Van Gestel, 2012):

- How could bioavailability be included in ERA schemes without losing protectivity?
- Should the information gained in sub-individual studies be involved in regulatory risk assessment and if yes, how?
- How can enchytraeids be established in higher-tier tests such as TMEs or field tests—and would it be useful to standardize gnotobiotic test systems?
- How can relevant protection goals for enchytraeids be defined, both for their functional roles and for their diversity (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017)?
- How much do interactions with other soil organisms account when discussing the effects of pesticides on enchytraeids (Menezes-Oliveira et al., 2011)?

Pesticides are just one factor potentially affecting enchytraeids in the field. Almost no information is available on the interactive effects on enchytraeids of pesticides applied together (as mixtures) or as part of normal agricultural practice. Similarly, no information could be found on the effects of adjuvants. In temperate regions, crop plants are often treated with several formulations of pesticides per season, while under tropical conditions more than 10 applications may occur within the same period (Waichman et al., 2002). Such realistic scenarios have never been investigated regarding their influence on enchytraeids. Almost the same is true regarding the interaction between pesticides and other agricultural practices (e.g., soil compaction due to the use of heavy machinery; Beylich et al., 2010). Finally, interactions between pesticide use and changing environmental conditions (in particular, temperature and soil moisture) in the context of Global Climate Change, could be important. Actually, based on studies with *E. albidus* and nonylphenol (included in some fungicide formulations), Silva et al. (2016) recommended to include environmental factors such as salinization in standard test procedures. Surely, such modifications should be investigated more intensively.

Enchytraeids are potentially affected by pesticides world-wide, but so far these organisms have been neglected in most parts of the world, with the notable exception of Brazil (Niva et al., 2016). Therefore, considering their key roles in soils, laboratory, semi-field, and field studies are urgently needed in most parts of the world—of course only in those in which enchytraeids are abundant enough to be used as indicators (e.g., not in permanently dry regions).

As mentioned above, the regulations for the risk assessment of pesticides in Europe are currently under discussion (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2017). Enchytraeids are one out of seven soil organism groups for which specific protection goals have been defined—and they are quite detailed regarding the magnitude and duration of effects. However, it is not clear whether the expectations of this document can be fulfilled by the existing test methods. While this is probably no problem for laboratory tests (especially when including recent ideas on life-cycling testing; Bicho et al., 2015), there is surely work to be done on higher-tier testing. Regarding semi-field tests (e.g., TMEs), the main problem is the formal standardization of existing approaches (Schaeffer et al., 2010). In contrast, there is no detailed proposal for an enchytraeid field test, despite the fact that the well-known earthworm field test (International Organization for Standardization, 1999) could probably be combined with enchytraeid samplings as described in monitoring guidelines (International Organization for Standardization, 2007).

CONCLUSIONS

The information provided in this paper can be summarized as follows:

- The existing information on the effects of pesticides on enchytraeids in agro-ecosystems has been compiled.
- Few pesticides have been tested in comparison to the total number of PPPs commonly used in agroecosystems.
- Enchytraeidae are common soil invertebrates, which at least in some soils can occur in high numbers and diversity. However, due to their small size and for taxonomic reasons, they are less considered in ecology and ecotoxicology than, for example, their larger relatives, earthworms.
- For these reasons, they are not regularly tested in the context of pesticide environmental risk assessment. However, standard laboratory tests were developed and their inclusion in existing semi-field and field test methods was proposed and seems to

be possible with limited efforts. They they are one out of seven soil organism groups for which specific protection goals (SPGs) have recently been formulated.
- There are good reasons why they should be used more regularly: laboratory tests do require not much space or amounts of soil. A wide range of standard test methods is available, and more are in the making (e.g., multi-generation tests).
- Even more obvious are advantages in higher-tier tests: in TME tests complex interactions with other soil organisms under a wide range of environmental conditions can be studied with relatively low efforts in comparison to field tests.
- Independently from the test level, enchytraeids are very useful for the study of interactions between pesticides and biotic or abiotic stress factors (mainly soil properties but also anthropogenic factors such as Global Climate Change).
- Although enchytraeid ecology and ecotoxicology is clearly biased toward studies in European temperate conditions, good examples of their use as test organisms and bioindicators are also present in Mediterranean and tropical environments; this should motivate researchers worldwide to dedicate attention to these important but overlooked key players of soil food webs.

AUTHOR CONTRIBUTIONS

The authors did equally contribute to the design, data acquisition, data interpretation, drafting, and critical reviewing of this work. JR, RS, and CP approved this final version and agree to be accountable for all aspects presented here.

ACKNOWLEDGMENTS

We thank Nicola Böffinger for her help identifying and collecting the literature used in this review and Stephan Jänsch for his critical comments on earlier versions of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2017.00020/full#supplementary-material

REFERENCES

Abrahamsen, G., and Thompson, W. N. (1979). A long term study of the enchytraeid (Oligochaeta) fauna of a mixed coniferous forest and the effects of urea fertilization. *Oikos* 32, 318–327. doi: 10.2307/3544742

Achazi, R. K., Fröhlich, E., Henneken, M., and Pilz, C. (1999). The effect of soil from former irrigation fields and of sewage sludge on dispersal activity and colonizing success of the annelid *Enchytraeus crypticus* (Enchytraeidae, Oligochaeta). *Newsl. Enchytr.* 6, 117–126.

American Society for Testing and Materials (1993). "Standard guide for conducting a terrestrial soil-core microcosm test," in *Annual Book*, Vol. 1197 (West Conshohocken, PA: ASTM), 546–557.

American Society for Testing and Materials (2004). *Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm Eisenia fetida and the Enchytraeid Potworm Enchytraeus albidus.* West Conshohocken, PA: ASTM. ASTM Guideline No. E 1676–97.

Amorim, M. J. B., Novais, S. C., van der Ven, K., Vandenbrouck, T., Soares, A. M. V. M., and de Coen, W. (2011). Development of a microarray for *Enchytraeus albidus* (Oligochaeta): preliminary tool with diverse applications. *Environ. Toxicol. Chem.* 30, 1395–1402. doi: 10.1002/etc.512

Amorim, M. J. B., Novais, S., Römbke, J., and Soares, A. M. V. M. (2008a). Avoidance test with *Enchytraeus albidus* (Enchytraeidae): effects of different exposure time and soil properties. *Environmental Pollution* 155, 112–116. doi: 10.1016/j.envpol.2007.10.028

Amorim, M. J. B., Novais, S., Römbke, J., and Soares, A. M. V. M. (2008b). *Enchytraeus albidus* (Enchytraeidae): a test organism in a standardised avoidance test? Effects of different chemical substances. *Environ. Int.* 34, 363–371. doi: 10.1016/j.envint.2007.08.010

Amorim, M. J. B., Römbke, J., Scheffczyk, A., and Soares, A. M. V. M. (2005a). Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide phenmedipham. *Chemosphere* 61, 1102–1114. doi: 10.1016/j.chemosphere.2005.03.048

Amorim, M. J. B., Römbke, J., and Soares, A. M. V. M. (2005b). Avoidance behaviour of *Enchytraeus albidus*: effects of benomyl, carbendazim, phenmedipham and different soil types. *Chemosphere* 59, 501–510. doi: 10.1016/j.chemosphere.2005.01.057

Amorim, M. J. B., Sousa, J. P., Nogueira, A. J. A., and Soares, A. M. V. M. (1999). Comparison of chronic toxicity of Lindane (gamma-HCH) to *Enchytraeus albidus* in two soil types: the influence of soil pH. *Pedobiologia* 43, 635–640.

Amorim, M. J., Sousa, J. P., Nogueira, A. J. A., and Soares, A. M. V. M. (2002). Bioavailability and Toxicokinetics of 14C-Lindane (γ-HCH) in the Enchytraeid *Enchytraeus albidus* in two soil types: the aging effect. *Arch. Environ. Contamin. Toxicol.* 43, 221–228. doi: 10.1007/s00244-002-1162-y

Amorim, M., Soares, A. M. V. M., and Römbke, J. (2005). Comparison of the influence of an artificial and a natural soil on the behaviour of *Enchytraeus albidus* – laboratory tests. *Proc. Eston. Acad. Sci. Biol. Ecol.* 54, 335–341.

An, Y.-J., and Yang, C.-Y. (2009). *Fridericia peregrinabunda* (Enchytraeidae) as a new test species for soil toxicity assessment. *Chemosphere* 77, 325–329. doi: 10.1016/j.chemosphere.2009.07.013

Arrate, J., Angel, R. P., and Martinez-Madrid, M. (2002). Effects of three chemicals on the survival and reproduction of the oligochaete worm *Enchytraeus coronatus* in chronic toxicity tests. *Pedobiologia* 46, 136–149. doi: 10.1078/0031-4056-00120

Assis, O. (2015). *Enchytraeids (Enchytraeidae, Oligochaeta) as Indicators of Soil Management and Ecotoxicological Tests*. Dissertation, Universidade Tecnológica Federal do Paraná, Curitiba.

Associação Brasileira de Normas Técnicas (2012). *Qualidade do Solo - Efeitos de Poluentes em Enchytraeidae (Enchytraeus sp.) - Determinação de Efeitos sobre a Reprodução e Sobrevivência*. Rio de Janeiro: ABNT, No. ISO: 16387.

Augustsson, A. K., and Rundgren, S. (1998). The enchytraeid *Cognettia sphagnetorum* in risk assessment: advantages and disadvantages. *Ambio* 27, 62–69.

Bandow, C., Coors, A., and Römbke, J. (2013). *Enchytraeus bigeminus* (Enchytraeidae, Oligochaeta) as a new candidate for ecotoxicological laboratory tests. *Soil Organisms* 85, 103–112.

Bandow, C., Ng, E. L., Schmelz, R. M., Sousa, J., Paulo, and Römbke, J. (2016). A TME study with the fungicide pyrimethanil combined with different moisture regimes: effects on enchytraeids. *Ecotoxicology* 25, 213–224. doi: 10.1007/s10646-015-1581-y

Banerjee, B. D., Seth, V., and Ahmed, R. S. (2001). Pesticide-induced oxidative stress: perspective and trends. *Rev. Environ. Health* 16, 1–40. doi: 10.1515/REVEH.2001.16.1.1

Barth, N., Brandtner, W., Cordsen, E., Dann, T., Emmerich, K.-H., Feldhaus, D., et al. (2000). "Boden-Dauerbeobachtung – Einrichtung und Betrieb von Boden-Dauerbeobachtungsflächen," in *Bodenschutz. Kennziffer 9152,* eds D. Rosenkranz, G. Bachmann, W. König, and G. Einsele (Berlin: Erich Schmidt Verlag), 1–127.

Bengtsson, G., and Rundgren, S. (1982). Population density and species number of enchytraeids in coniferous forest soils polluted by a brass mill. *Pedobiologia* 24, 211–218.

Beylich, A., and Graefe, U. (2009). Investigations of annelids at soil monitoring sites in Northern Germany: reference ranges and time-series data. *Soil Organisms* 81, 175–196.

Beylich, A., Oberholzer, H.-R., Schrader, S., Höper, H., and Wilke, B.-M. (2010). Evaluation of soil compaction effects on soil biota and soil biological processes in soils. *Soil Tillage Res.* 109, 133–143. doi: 10.1016/j.still.2010.05.010

Bezchlebová, J., Černohlávková, J., Lána, J., Sochová, I., Kobetičová, K., and Hofman, J. (2007). Effects of toxaphene on soil organisms. *Ecotoxicol. Environ. Saf.* 68, 326–334. doi: 10.1016/j.ecoenv.2007.05.009

Bicho, R. C., Santos, F. C. F., Gonçalves, M. F. M., Soares, A. M. V. M., and Amorim, M. J. B. (2015). Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test—An optional multi-endpoint test with *Enchytraeus crypticus*. *Ecotoxicology* 24, 1053–1063. doi: 10.1007/s10646-015-1445-5

Bispo, A., Cluzeau, D., Creamer, R., Dombos, M., Graefe, U., Krogh, P., et al. (2009). Indicators for monitoring soil biodiversity. *Integr. Environ. Assess. Manag.* 5, 717–719. doi: 10.1897/IEAM-2009-064.1

Born, H. (1993). *Die Sukzession der Enchytraeen-Synusie (Annelida, Oligochaeta) eines Ruderalökosystems unter Natürlichen und Anthropogenen Einflüssen*. Dissertation, Universität Bremen, Bremen.

Brüggl, G. (1994). *Populationsentwicklung von Enchytraeus crypticus (Enchytraeidae, Oligochaeta) und Einfluss subletaler Pestizidbelastungen unter Laborbedingungen.* Dissertation, University of Osnabrück, Osnabrück.

Bruns, E., Egeler, P., Roembke, J., Scheffczyk, A., and Spoerlein, P. (2001). Bioaccumulation of lindane and hexachlorobenzene by the oligochaetes *Enchytraeus luxuriosus* and *Enchytraeus albidus* (Enchytraeidae, Oligochaeta, Annelida). *Hydrobiologia* 463, 185–196. doi: 10.1023/A:1013159810067

Brussaard, L. (2012). "Ecosystem services provided by the soil biota," in *Soil Ecology and Ecosystem Services*, eds D. H. Wall, R. D. Bardgett, V. Behan-Pelletier, J. E. Herrick, H. Jones, K. Ritz, J. Six, D. R. Strong, and W. H. van der Putten (Oxford: Oxford University Press), 45–58.

Burkhardt, U., Russell, D. J., Buryn, R., Decker, P., Döhler, M., Höfer, H., et al. (2014). The Edaphobase Project of GBIF-Germany – a new online soil- organism zoological data warehouse. *Appl. Soil Ecol.* 83, 5–12. doi: 10.1016/j.apsoil.2014.03.021

Burrows, L. A., and Edwards, C. A. (2004). The use of integrated soil microcosms to assess the impact of carbendazim on soil ecosystems. *Ecotoxicology* 13, 143–161. doi: 10.1023/B:ECTX.0000012411.14680.21

Castro-Ferreira, M. P., Roelofs, D., van Gestel, C., Verweij, R. A., Soares, A. M. V. M., and Amorim, M. J. B. (2012). *Enchytraeus crypticus* as model species in soil ecotoxicology. *Chemosphere* 87, 1222–1227. doi: 10.1016/j.chemosphere.2012.01.021

Chalupský, J. Jr. (1989). The influence of Zeazin 50 on Enchytraeidae (Oligochaeta) in an apple orchard soil. *Pedobiologia* 33, 361–371.

Chalupský, J. Jr., and Lepš, J. (1985). The spatial pattern of Enchytraeidae (Oligochaeta). *Oecologia* 68, 153–157. doi: 10.1007/BF00379488

Chelinho, S., Domene, X., Campana, P., Andrés, P., Römbke, J., Sousa, J., et al. (2014). Toxicity of phenmedipham and carbendazim to *Enchytraeus crypticus* and *Eisenia andrei* (Oligochaeta) in Mediterranean soils. *J. Soils Sediments* 14, 584–599. doi: 10.1007/s11368-013-0818-8

Chelinho, S., Lopes, I., Natal-da-Luz, T., Domene, X., Tenorio Nunes, M. E., Espíndola, E. L. G., et al. (2012). Integrated ecological risk assessment of pesticides in tropical ecosystems: a case study with carbofuran in Brazil. *Environ. Toxicol. Chem.* 31, 437–445. doi: 10.1002/etc.738

Christensen, B., and Jensen, L. O. (1995). "Toxicity of pesticides to *Enchytraeus bigeminus*" in *Effects of pesticides on meso- and microfauna in soil,* ed H. Løkke (Copenhague: Ministry of Environment and Energy), 33–38.

Collado, R., Hass-Cordes, E., and Schmelz, R. M. (2012). Microtaxonomy of fragmenting *Enchytraeus* species using molecular markers, with a comment on species complexes in enchytraeids. *Turk. J. Zool.* 36, 85–94. doi: 10.3906/zoo-1002-70

Collado, R., Schmelz, R. M., Moser, T., and Römbke, J. (1999). Enchytraeid reproduction test (ERT): sublethal responses of two *Enchytraeus* species (Oligochaeta) to toxic chemicals. *Pedobiologia* 43, 625–629.

Commission of the European Communities (1991). Council directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. *Official J. Eur. Commun.* L 230/1.

Cortet, J., Gomot-De Vauflery, A., Poinsot-Balaguer, N., Gomot, L., Texier, C., and Cluzeau, D. (1999). The use of invertebrate soil fauna in monitoring pollutant effects. *Eur. J. Soil Biol.* 35, 115–134. doi: 10.1016/S1164-5563(00)00116-3

de Amorim, M. J., Sousa, J. P., Nogueira, A. J. A., and Soares, A. M. V. M. (2002). Bioaccumulation and elimination of 14C-lindane by *Enchytraeus albidus* in artificial (OECD) and a natural soil. *Chemosphere* 49, 323–329. doi: 10.1016/S0045-6535(02)00322-3

Didden, W. A. M. (1990). Involvement of Enchytraeidae (Oligochaeta) in soil structure evolution in agricultural fields. *Biol. Fertil. Soils* 9, 152–158. doi: 10.1007/BF00335799

Didden, W. A. M. (1993). Ecology of terrestrial Enchytraeidae. *Pedobiologia* 37, 2–29.

Didden, W. A. M., Fründ, H. L., and Graefe, U. (1997). "Enchytraeids," in *Fauna in Soil Ecosystems - Recycling Processes, Nutrient Fluxes and Agricultural Production*, ed G. Benckiser (Giessen: M. Dekker, Inc.), 135–172.

Didden, W., and Römbke, J. (2001). Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. *Ecotoxicol. Environ. Saf.* 50, 25–43. doi: 10.1006/eesa.2001.2075

Dormidontova, G. N. (1973). The effect of an insecticide on soil forming fauna (Arthropoda, Oligochaeea). *Pedobiologia* 13, 123–139.

Dózsa-Farkas, K. (1991). New enchytraeid species found very deep in soils of a hornbeam and oak forest in Hungary (Oligochaeta, Enchytraeidae). *Acta Zool. Hung.* 37, 21–25.

European Chemicals Agency (2014). *Guidance on Information Requirements and Chemical Safety Assessment*, Vol. 5, Helsinki: European Chemicals Agency.

Edwards, C. A., and Lofty, J. R. (1971). "Nematicides and the soil fauna," in *Proceedings of the Sixth British Insecticide and Fungicide*, Vol. 1 (Brighton).

Edwards, C. A., Thompson, A. R., and Beynon, K. I. (1968). Some effects of chlorfenvinphos, an organophosphorus insecticide on populations of soil animals. *Rev. Ecol. Biol. Sol.* 1, 199–224.

EFSA Panel on Plant Protection Products and their Residues (PPR), Ockleford, C., Adriaanse, P., Berny, P., Brock, T., Duquesne, S., et al. (2017). Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. *EFSA J.* 15:e4690. doi: 10.2903/j.efsa.2017.4690

European Plant Protection Organisation (2003). "Chapter 8: Soil organisms and functions," in *Environmental Risk Assessment Scheme of Plant Protection Products,* Vol. 33 (Paris: EPPO Bulletin), 195–209.

European Commission (2009). *Regulation (EC) 1107/2009 of the European Parliament and of the Council of 21 October 2009 Concerning the Placing of Plant Protection Products on the Market and repealing Council Directives 79/117/EEC and 91/414/EEC*, Vol. 309. Brussels: Official Journal of the European Union.

Ferreira, M. P. C., Roelofs, D., van Gestel, C. A. M., Amorim, M. J. B., and Soares, A. (2010). Ultra-high Throughput TRANScryptOME Sequencing of *Enchytraeus crypticus* - Innovative Tool for Stress Response Assessment. *Comp. Biochem. Physiol. Mol. Integr. Physiol.* 157, S32–S32. doi: 10.1016/j.cbpa.2010.06.092

Förster, B., Garcia, M., Francimari, O., and Römbke, J. (2006). Effects of carbendazim and lambda-cyhalothrin on soil invertebrates and leaf litter decomposition in semi-field and field tests under tropical conditions (Amazonia, Brazil). *Eur. J. Soil Biol.* 42, 171–179. doi: 10.1016/j.ejsobi.2006.07.011

Förster, B., van Gestel, C., Koolhaas, J. E., Nentwig, G., Rodrigues, J. M. L., and Sousa, J. P. (2004). Ring-testing and field-validation of a terrestrial model ecosystem (TME)–an instrument for testing potentially harmful substances: effects of carbendazim on organic matter breakdown and soil fauna feeding activity. *Ecotoxicology* 13, 129–141. doi: 10.1023/B:ECTX.0000012410.99020.97

Frampton, G. K., Jänsch, S., Scott-Fordsmand, J. J., Römbke, J., and van den Brink, P. J. (2006). Effects of pesticides on soil invertebrates in laboratory studies: a review and analysis using species sensitivity distributions. *Environ. Toxicol. Chem.* 25, 2480–2489. doi: 10.1897/05-438R.1

Friberg, H., Fayolle, L., Edel-Hermann, V., Gautheron, N., and Steinberg, C. F. C. (2009). Response of *Rhizoctonia solani* to soil faunal grazing and organic amendments - different from general microbial dynamics. *IOBC/WPRS Bull.* 42, 63–67.

Gomes, S. I. L., Novais, S. C., Soares, A. M. V. M., and Amorim, M. J. B. (2011). Effects of soil properties and time of exposure on gene expression of *Enchytraeus albidus* (Oligochaeta). *Soil Biol. Biochem.* 43, 2078–2084. doi: 10.1016/j.soilbio.2011.06.006

Gomes, S. I. L., Soares, A. M. V. M., and Amorim, M. J. B. (2015). Changes in cellular energy allocation in *Enchytraeus crypticus* exposed to copper and silver—linkage to effects at higher level (reproduction). *Environ. Sci. Pollut. Res.* 22, 14241–14247. doi: 10.1007/s11356-015-4630-4

Gonçalves, M. F. M., Bicho, R. C., Rêma, A., Soares, A. M. V. M., Faustino, A., and Amorim, M. J. B. (2015). Development of an embryotoxicity test for *Enchytraeus crypticus*–The effect of Cd. *Chemosphere* 139, 386–392. doi: 10.1016/j.chemosphere.2015.07.021

Graefe, U. (1993). Die Gliederung von Zersetzergesellschaften für die standortsökologische Ansprache. *Mitteilungen der Deutschen Bodenkundlichen Gesellschaft* 69, 95–98.

Graefe, U., and Beylich, A. (2003). "Critical values of soil acidification for annelid species and the decomposer community," in *Newsletter on Enchytraeidae,* Vol. 8, eds W. Didden, and P. Van Vliet (Wageningen: Department of Soil Quality, Wageningen University), 51–55.

Graefe, U., and Schmelz, R. M. (1999). "Indicator values, strategy types and life forms of terrestrial Enchytraeidae and other microannelids," in *Newsletter on Enchytraeidae,* Vol. 6, eds R. Schmelz, and K. Sühlo (Osnabrück: Universitätsverlag Rasch), 59–67.

Hartnik, T., Sverdrup, L. E., and Jensen, J. (2008). Toxicity of the pesticide alpha-cypermethrin to four soil nontarget invertebrates and implications for risk assessment. *Environ. Toxicol. Chem.* 27, 1408–1415. doi: 10.1897/07-385.1

Heungens, A. (1968). The influence of DBCP on the soil fauna in Azalea culture. *Pedobiologia* 8, 281–288.

Hopkin, S. P. (1989). *Ecophysiology of Metals in Terrestrial Invertebrates.* New York, NY: Elsevier Applied Science.

Howcroft, C. F., Amorim, M. J. B., Gravato, C., Guilhermino, L., and Soares, A. M. V. M. (2009). Effects of natural and chemical stressors on *Enchytraeus albidus*: can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? *Environ. Int.* 35, 318–324. doi: 10.1016/j.envint.2008.08.004

Howcroft, C. F., Gravato, C., Amorim, M. J. B., Novais, S. C., Soares, A. M. V. M., and Guilhermino, L. (2011). Biochemical characterization of cholinesterases in *Enchytraeus albidus* and assessment of *in vivo* and *in vitro* effects of different soil properties, copper and phenmedipham. *Ecotoxicology* 20, 119–130. doi: 10.1007/s10646-010-0562-4

International Organization for Standardization (1998). *Soil Quality–Effects of Pollutants on Earthworms (Eisenia fetida). Part 2: Determination of Effects on Reproduction.* Geneva: ISO 11268–2.

International Organization for Standardization (1999). *Soil Quality – Effects of Pollutants on Earthworms - Part 3: Guidance on the Determination of Effects in Field Situations.* Geneva: ISO 11268–3.

International Organization for Standardization (2004). *Soil Quality – Effects of Pollutants on Enchytraeidae (Enchytraeus sp.). Determination of Effects* on *Reproduction* and *Survival*. Geneva: ISO 16387.

International Organization for Standardization (2007). *Soil Quality – Sampling of Soil Invertebrates Part 3: Sampling and Soil Extraction of Enchytraeids.* Geneva: ISO 23611–3.

International Organization for Standardization (2008). *Soil Quality – Avoidance Test for Evaluating the Quality of Soils and the Toxicity of Chemicals. Test with Earthworms (Eisenia fetida/andrei).* Geneve: ISO 17512–1.

Jänsch, S., Frampton, G. K., Römbke, J., Van den Brink, P. J., and Scott-Fordsmand, J. J. (2006). Effects of pesticides on soil invertebrates in model ecosystem and field studies: a review and comparison with laboratory toxicity data. *Environ. Toxicol. Chem.* 25, 2490–2501. doi: 10.1897/05-439R.1

Jänsch, S., Römbke, J., and Didden, W. (2005). The use of enchytraeids in ecological classification and assessment concepts. *Ecotoxicol. Environ. Saf.* 62, 266–277. doi: 10.1016/j.ecoenv.2004.10.025

Jarratt, N., and Thompson, H. (2009). *Comparison between the Sensitivity of Enchytraeids and Lumbricidae to Chemicals, in Particular Plant Protection Products.* York, UK: EFSA Supporting Publications.

Jensen, J., and Scott-Fordsmand, J. J. (2012). Ecotoxicity of the veterinary pharmaceutical ivermectin tested in a soil multi-species (SMS) system. *Environ. Pollut.* 171, 133–139. doi: 10.1016/j.envpol.2012.07.014

Kattwinkel, M., Liess, M., Arena, M., Bopp, S., Streissl, F., and Römbke, J. (2015). Recovery of aquatic and terrestrial populations in the context of European pesticide risk assessment. *Environ. Rev.* 23, 382–394. doi: 10.1139/er-2015-0013

Kitazawa, Y., and Kitazawa, T. (1980). "Influence of application of a fungicide, an insecticide, and compost upon soil biotic community," in *Soil Biology as Related to Land Use Practices,* ed D. L. Dindal (Washington, DC: Office of Pesticide and Toxic Substances, Environmental Protection Agency), 94–99.

Knacker, T., Van Gestel, C. A. M., Jones, S. E., Soares, A. M. V. M., Schallnaß, H.-J., Förster, B., et al. (2004). Ring-testing and field validation of a terrestrial model ecosystem (TME) – an instrument for testing potentially harmful substances: conceptual approach and study design. *Ecotoxicology* 13, 5–23. doi: 10.1023/B:ECTX.0000012402.38786.01

Kobetičová, K., Hofman, J., and Holoubek, I. (2009). Avoidance response of *Enchytraeus albidus* in relation to carbendazim ageing. *Environ. Pollut.* 157, 704–706. doi: 10.1016/j.envpol.2008.09.032

Kremen, C. (2005). Managing ecosystem services: what do we need to know about their ecology? *Ecol. Lett.* 8, 468–479. doi: 10.1111/j.1461-0248.2005.00751.x

Kristufek, V., and Ruzicka, V. (1995). "Herbicide effects on the reproduction of *Enchytraeus crypticus* (Oligochaeta)," in *8th International Bioindicators Symposium* (Ceske Budejovice).

Kuperman, R. G., Amorim, M. J. B., Römbke, J., Lanno, R., Checkai, R. T., Dodard, S. G., et al. (2006). Adaptation of the enchytraeid toxicity test for use with natural soil types. *Eur. J. Soil Biol.* 42, 234–243. doi: 10.1016/j.ejsobi.2006.07.028

Kuperman, R. G., Simini, M., Phillips, C. T., and Checkai, R. T. (1999). Comparison of malathion toxicity using enchytraeid reproduction test and earthworm toxicity test in different soil types. *Pedobiologia* 43, 630–634.

Laakso, J., and Setälä, H. (1999). Sensitivity of primary production to changes in the architecture of belowground food webs. *Oikos* 87, 57–64. doi: 10.2307/3546996

Lagerlöf, J., Andren, O., and Paustian, K. (1989). Dynamics and contribution to carbon flows of Enchytraeidae (Oligochaeta) under four cropping systems. *J. Appl. Ecol.* 26, 183–199. doi: 10.2307/2403660

Lavtižar, V., Berggren, K., Trebše, P., Kraak, M. H. S., Verweij, R. A., and van Gestel, C. (2016). Comparative ecotoxicity of chlorantraniliprole to non-target soil invertebrates. *Chemosphere* 159, 473–479. doi: 10.1016/j.chemosphere.2016.06.036

Leitão, S., José Cerejeira, M., Van den Brink, P. J., and Sousa, J. P. (2014). Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. *Appl. Soil Ecol.* 76, 124–131. doi: 10.1016/j.apsoil.2013.12.013

Lock, K., and Janssen, C. R. (2002). Multi-generation toxicity of zinc, cadmium, copper and lead to the potworm *Enchytraeus albidus*. *Environ. Pollut.* 117, 89–92. doi: 10.1016/S0269-7491(01)00156-7

Lock, K., Schamphelaere, K. A. C., de, and Janssen, C. R. (2002). The effect of lindane on terrestrial invertebrates. *Arch. Environ. Contaminat. Toxicol.* 42, 217–221. doi: 10.1007/s00244-001-0009-2

Løkke, H., and van Gestel, C. A. M. (1998). *Handbook of Soil Invertebrate Toxicity Tests.* Chichester, UK: John Wiley & Sons.

Loureiro, S., Amorim, M. J. B., Campos, B., Rodrigues, S. M. G., and Soares, A. M. V. M. (2009). Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. *Environ. Pollut.* 157, 625–636. doi: 10.1016/j.envpol.2008.08.010

Maraldo, K., and Holmstrup, M. (2010). Enchytraeids in a changing climate: a mini-review. *Pedobiologia* 53, 161–167. doi: 10.1016/j.pedobi.2009.10.003

Marinissen, J. C. Y., and Didden, W. A. M. (1997). Influence of the enchytraeid worm *Buchholzia appendiculata* on aggregate formation and organic matter decomposition. *Soil Biol. Biochem.* 29, 387–390. doi: 10.1016/S0038-0717(96)00100-9

Martikainen, E. (1996). Toxicity of dimethoate to some soil animal species in different soil types. *Ecotoxicol. Environ. Saf.* 33, 128–136. doi: 10.1006/eesa.1996.0016

Martikainen, E., Haimi, J., and Ahtiainen, J. (1998). Effects of dimethoate and benomyl on soil organisms and soil processes–a microcosm study. *Appl. Soil Ecol.* 9, 381–387. doi: 10.1016/S0929-1393(98)00093-6

Martin, N. A. (1975). Effect of four insecticides on the pasture ecosystem: IV. Enchytraeidae and Diptera larvae heat-extracted in water-filled funnels. *N.Z. J. Agric. Res.* 18, 313–315. doi: 10.1080/00288233.1975.10423650

Martinsson, S., Rota, E., and Erséus, C. (2015). Revision of *Cognettia* (Clitellata, Enchytraeidae): re-establishment of *Chamaedrilus* and description of cryptic species in the *sphagnetorum* complex. *Syst. Biodivers.* 13, 257–277. doi: 10.1080/14772000.2014.986555

McColl, H. P. (1984). Nematicides and field population of enchytraeids and earthworms. *Soil Biol. Biochem.* 16, 139–143.

Millennium Ecosystem Assessment (2005). *Ecosystems and Human Well-being: Synthesis*. Washington, DC: Island Press.

Menezes-Oliveira, V. B., Scott-Fordsmand, J. J., Rocco, A., Soares, A. M. V. M., and Amorim, M. J. B. (2011). Interaction between density and Cu toxicity for *Enchytraeus crypticus* and *Eisenia fetida* reflecting field scenarios. *Sci. Total Environ.* 409, 3370–3374. doi: 10.1016/j.scitotenv.2011.04.033

Menezes-Oliveira, V. B., Scott-Fordsmand, J., Soares, A. M. V. M., and Amorim, M. J. B. (2014). Development of ecosystems to climate change and the interaction with pollution—Unpredictable changes in community structures. *Appl. Soil Ecol.* 75, 24–32. doi: 10.1016/j.apsoil.2013.10.004

Mercurio, S. D. (2017). *Understanding Toxicology - A Biological Approach*. Burlington, MA: Jones & Bartlett Publishers.

Morgan, E., and Knacker, T. (1994). The role of laboratory terrestrial model ecosystems in the testing of potentially harmful substances. *Ecotoxicology* 3, 213–223. doi: 10.1007/BF00117989

Moser, T., and Römbke, J. (2007). "Enchytraeid species assemblage and dominance spectrum in soils from four different European sites investigated in Terrestrial Model Ecosystems (TME) and in the field," in *Newsletter on Enchytraeidae No. 10: Proceedings of the 7th International Symposium on Enchytraeidae* (Brno: Folia Facultatis Scientiarum Naturalium Universitatis Masarykianae Brunensis, Biologia), 141–155.

Moser, T., Römbke, J., Schallnass, H.-J., and van Gestel, C. (2007). The use of the multivariate Principal Response Curve (PRC) for community level analysis: a case study on the effects of carbendazim on enchytraeids in Terrestrial Model Ecosystems (TME). *Ecotoxicology* 16, 573–583. doi: 10.1007/s10646-007-0169-6

Moser, T., van Gestel, C., Jones, S. E., Koolhaas, J. E., Rodrigues, J. M. L., and Römbke, J. (2004). Ring-testing and field-validation of a Terrestrial Model Ecosystem (TME) – An instrument for testing potentially harmful substances: effects of carbendazim on enchytraeids. *Ecotoxicology* 13, 89–103. doi: 10.1023/B:ECTX.0000012407.42358.3e

Mothes-Wagner, U., Reitze, H. K., and Seitz, K.-A. (1992). Terrestrial multispecies toxicity testing: 1. Description of the multispecies assemblage. *Chemosphere* 24, 1653–1667. doi: 10.1016/0045-6535(92)90408-J

Mulder, C., Boit, A., Bonkowski, M., de Ruiter, P. C., Mancinelli, G., van der Heijden, M. G. A., et al. (2011). A belowground perspective on Dutch agroecosystems: how soil organisms interact to support ecosystem services. *Adv. Ecol. Res.* 44, 277–357. doi: 10.1016/B978-0-12-374794-5.00005-5

Natal-da-Luz, T., Moreira-Santos, M., Ruepert, C., Castillo, L. E., Ribeiro, R., and Sousa, J. P. (2012). Ecotoxicological characterization of a tropical soil after diazinon spraying. *Ecotoxicology* 21, 2163–2176. doi: 10.1007/s10646-012-0970-8

Nielsen, C. O. (1954). Studies on Enchytraeidae: 3. The Micro-Distribution of Enchytraeidae. *Oikos* 5, 167–178. doi: 10.2307/3565158

Nielsen, C. O. (1955). Studies on Enchytraeidae 5. Factors causing seasonal fluctuations in numbers. *Oikos* 6, 153–169. doi: 10.2307/3564852

Nielsen, C. O., and Christensen, B. (1959). The Enchytraeidae. Critical revision and taxonomy of European species (Studies on Enchytraeidae VII). *Nat. Jutlandica* 8–9, 1–160.

Nielsen, C. O., and Christensen, B. (1961). "The Enchytraeidae," in *Critical Revision and Taxonomy of European Species. Supplement 1,* Vol. 10 (Aarhus: Natura Jutlandica), 1–23.

Nielsen, C. O., and Christensen, B. (1963). "The Enchytraeidae," in *Critical Revision and Taxonomy of European Species. Supplement 2*, Vol. 10 (Aarhus: Natura Jutlandica), 1–19.

Niva, C. C., Niemeyer, J. C., Rodrigues da Silva Júnior, F. M., Tenório Nunes, M. E., de Sousa, D. L., Silva Aragão, C. W., et al. (2016). Soil Ecotoxicology in Brazil is taking its course. *Environ. Sci. Poll.* Res. 23, 363–378. doi: 10.1007/s11356-016-6597-1

Novais, S. C., and Amorim, M. J. B. (2013). Changes in cellular energy allocation in *Enchytraeus albidus* when exposed to dimethoate, atrazine, and carbendazim. *Environ. Toxicol. Chem.* 32, 2800–2807. doi: 10.1002/etc.2368

Novais, S. C., and Amorim, M. J. B. (2014). Normal operating range (NOR) in *Enchytraeus albidus* – Transcriptional responses to control conditions. *Appl. Soil Ecol.* 85, 1–10. doi: 10.1016/j.apsoil.2014.08.005

Novais, S. C., Arrais, J., Lopes, P., Vandenbrouck, T., de Coen, W., and Roelofs, D. (2012a). *Enchytraeus albidus* microarray: enrichment, design, annotation and database (EnchyBASE). *PLoS ONE* 7:e34266. doi: 10.1371/journal.pone.0034266

Novais, S. C., de Coen, W., and Amorim, M. J. B. (2012b). Gene expression responses linked to reproduction effect concentrations (EC 10, 20, 50, 90) of dimethoate, atrazine and carbendazim, in *Enchytraeus albidus*. *PLoS ONE* 7: e36068. doi: 10.1371/journal.pone.0036068

Novais, S. C., Gomes, N. C., Soares, A. M. V. M., and Amorim, M. J. B. (2014). Antioxidant and neurotoxicity markers in the model organism *Enchytraeus albidus* (Oligochaeta): mechanisms of response to atrazine, dimethoate and carbendazim. *Ecotoxicology* 23, 1220–1233. doi: 10.1007/s10646-014-1265-z

Novais, S. C., Howcroft, C. F., Carreto, L., Pereira, P. M., Santos, M. A. S., de Coen, W., et al. (2012c). Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors at different exposure periods. *Ecotoxicology* 21, 213–224. doi: 10.1007/s10646-011-0780-4

Novais, S. C., Soares, A. M. V. M., and Amorim, M. J. B. (2010). Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing? *Chemosphere* 79, 233–237. doi: 10.1016/j.chemosphere.2010.01.011

Organization for Economic Co-operation and Development (1984). *Guideline for Testing of Chemicals No. 207. Earthworm Acute Toxicity test.* Paris: Organization for Economic Co-operation and Development.

Organisation for Economic Co-operation and Development (2004). *Guideline for the Testing of Chemicals, No. 220. Enchytraeid Reproduction Test.* Paris: Organisation for Economic Co-operation and Development.

Organisation for Economic Co-operation and Development (2010). *Bioaccumulation in Terrestrial Oligochaetes. Guideline for the testing of chemicals 317.* Paris: Organisation for Economic Co-operation and Development.

Orgiazzi, A., Dunbar, M. B., Panagos, P., de Groot, G. A., and Lemanceau, P. (2015). Soil biodiversity and DNA barcodes: opportunities and challenges. *Soil Biol. Biochem.* 80, 244–250. doi: 10.1016/j.soilbio.2014.10.014

Peachey, J. E. (1963). Studies on the Enchytraeidae (Oligochaeta) of moorland soil. *Pedobiologia* 2, 81–95.

Peijnenburg, W., Capri, E., Kula, C., Liess, M., Luttik, R., Montforts, M., et al. (2012). Evaluation of exposure metrics for effect assessment of soil invertebrates. *Crit. Rev. Environ. Sci. Technol.* 42, 1862–1893. doi: 10.1080/10643389.2011.574100

Pelosi, C., and Römbke, J. (2016). Are Enchytraeidae (Oligochaeta, Annelida) good indicators of agricultural management practices? *Soil Biol. Biochem.* 100, 255–263. doi: 10.1016/j.soilbio.2016.06.030

Petersen, H., and Luxton, M. (1982). A comparative analysis of soil fauna populations and their role in decomposition processes. *Oikos* 39, 288–388. doi: 10.2307/3544689

Pokarzhevskii, A. D., Filimonova, Z. h. V., and Goryachev, O. A. (2003). Life-cycle length determines the differences in sensitivity to toxicants between Enchytraeid species. *Dokl. Biol. Sci.* 390, 256–258.

Popovici, I., Stan, G., Stefan, V., Tomescu, R., Dumea, A., Tarta, A., et al. (1977). The influence of Atrazine on soil fauna. *Pedobiologia* 17, 209–215.

Pryor, W. (1991). The antioxidant nutrients and disease prevention - what do we know and what do we need to find out? *Am. J. Clin. Nutr.* 53 (Suppl. 1), 391S–393S.

Purschke, G., Hagens, M., and Westheide, W. (1991). Ultrahistopathology of enchytraeid oligochaetes (Annelida) after exposure to pesticides—A means of identification of sublethal effects? *Comp. Biochem. Physiol. C Comp. Pharmacol.* 100C, 119–122. doi: 10.1016/0742-8413(91)90136-H

Puurtinen, H. M., and Martikainen, E. A. T. (1997). Effect of soil moisture on pesticide toxicity to an enchytraeid worm, *Enchytraeus* sp. *Arch. Environ. Contam. Toxicol.* 33, 34–41. doi: 10.1007/s002449900220

Römbke, J. (1988). *Die Enchytraeen eines Moderbuchenwaldes, ihre Rolle beim Streuabbau und ihre Reaktion auf Umweltbelastungen*. Dissertation, Frankfurt am Main.

Römbke, J. (1989). "*Enchytraeus albidus* (Oligochaeta, Annelida) as a test organism in terrestrial laboratory systems," in *Biological Monitoring of Exposure and the Response at the Subcellular Level to Toxic Substances, Archives of Toxicology,* eds

P. L. Chambers, C. M. Chambers, and H. Greim (Berlin; Heidelberg: Springer), 402–405.

Römbke, J. (1991). Umweltverhalten von Chemikalien in einem terrestrischen Ökosystemausschnitt: Effekte auf Enchytraeidae (Oligochaeta). *Verhandlungen Gesellschaft Ökologie* 19, 157.

Römbke, J. (2001). Auswirkungen zweier Umweltchemikalien auf die Enchytraeen eines Moderbuchenwalds. *Andrias* 15, 205–218.

Römbke, J. (2003). Ecotoxicological laboratory tests with enchytraeids: a review. *Pedobiologia* 47, 607–616. doi: 10.1078/0031-4056-00235

Römbke, J., Beck, L., Dreher, P., Hund-Rinke, K., Jänsch, S., Kratz, W., et al. (2002). *Entwicklung von Bodenbiologischen Bodengüteklassen für Acker- und Grünlandstandorte.* UBA-Texte 20/02. Federal Environment Agency.

Römbke, J., Dreher, P., Beck, L., Hammel, W., Hund, K., Knoche, H., et al. (2000). *Bodenbiologische Bodengüte-Klassen.* UBA-Texte 6/00. Federal Environment Agency.

Römbke, J., and Federschmidt, A. (1995). "Effects of the fungicide carbendazim on Enchytraeidae in laboratory and field tests," in *Newsletter on Enchytraeidae,* Vol. 4, ed R. Bauer (Vienna: University of Agriculture) 79–96.

Römbke, J., Jänsch, S., Höfer, H., Horak, F., Roß-Nickoll, M., Russell, D., et al. (2013). State of knowledge of enchytraeid communities in German soils as a basis for biological soil quality assessment. *Soil Organisms* 85, 123–146.

Römbke, J., and Knacker, T. (1989). Aquatic toxicity test for enchytraeids. *Hydrobiologia* 180, 235–242. doi: 10.1007/BF00027556

Römbke, J., Knacker, T., Förster, B., and Marcinkowski, A. (1994). "Comparison of effects of two pesticides on soil organisms in laboratory tests, microcosms and in the field," in *Ecotoxicology of Soil Organisms*, eds M. Donker, H. Eijsackers, and F. Heimbach (Chelsea, MI: Lewis Publisher), 229–240.

Römbke, J., and Moser, T. (1999). *Organisation and Performance of an International Ringtest for the Validation of the Enchytraeid Reproduction Test Vol. I and II.* UBA-Texte 4/99. Federal Environment Agency.

Römbke, J., and Moser, T. (2002). Validating the enchytraeid reproduction test: organisation and results of an international ringtest. *Chemosphere* 46, 1117–1140. doi: 10.1016/S0045-6535(01)00113-8

Römbke, J., Schmelz, R., and Knaebe, S. (2009). Field studies for the assessment of pesticides with soil mesofauna, in particular enchytraeids, mites and nematodes: design and first results. *Soil Organisms* 81, 234–264.

Rota, E., and Healy, B. (1999). A taxonomic study of some Swedish Enchytraeidae (Oligochaeta), with descriptions of four new species and notes on the genus *Fridericia*. *J. Natl. History* 33, 29–64. doi: 10.1080/00222939 9300461

Ruf, A., Beck, L., Römbke, J., and Spelda, J. (2000). Standortspezifische Erwartungswerte für die Gemeinschaftsstruktur ausgewählter Taxa der Bodenfauna als Bodenqualitätskriterium. *Berichte Naturwissenschaftlich-Medizinischen Vereins in Innsbruck* 87, 365–379.

Rutgers, M., Mulder, C., and Schouten, A. J. (eds.). (2008). *Soil Ecosystem Profiling in the Netherlands with Ten References for Biological Soil Quality*. RIVM-Report 607604009.

Rüther, U., and Greven, H. (1990). The effect of heavy metals on enchytraeids. 1. Uptake from an artificial substrate and influence on food preference. *Acta Biol. Benrodis* 2, 125–131.

Schaeffer, A., van den Brink, P. J., Heimbach, F., Hoy, S. P., de Jong, F. M. W., Römbke, J., et al. (2010). *Guidance from the SETAC Europe Workshop: Semi-field Methods for the Environmental Risk Assessment of Pesticides in Soil (PERAS)*. Boca Raton, FL: CRC Press.

Schärffenberg, B. (1950). Untersuchungen über die Bedeutung der Enchytraeiden als Humusbildner und Nematodenfeinde. - Z. Pflanzenkrankh. *Pflanzenschutz* 57, 183–191

Schlaghamerský, J. (2015). Short note on enchytraeid occurrence in deep layers of urban soils. *Soil Organisms* 87, 85–89.

Schmelz, R. M. (2003). Taxonomy of *Fridericia* (Oligochaeta, Enchytraeidae). Revision of species with morphological and biochemical methods. *Abhandlungen des Naturwissenschaftlichen Vereins in Hamburg* (Neue Folge) 38:415.

Schmelz, R. M., and Collado, R. (2010). A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta). *Soil Organisms* 82, 1–176.

Schmelz, R. M., and Collado, R. (2012). An updated checklist of currently accepted species of Enchytraeidae (Oligochaeta). *vTI Agric. Forest. Res.* 357, 67–87.

Schmidt, O., Curry, J. P., Dyckmans, J., Rota, E., and Scrimgeour, C. M. (2004). Dual stable isotope analysis (δ^{13}C and δ^{15}N) of soil invertebrates and their food sources. *Pedobiologia* 48, 171–180. doi: 10.1016/j.pedobi.2003.12.003

Schoch-Bösken, J., and Römbke, J. (1993). Bibliography of the Enchytraeidae (1950–1991). *Acta Biol. Benrodis* (Suppl. 1), 1–76.

Scholz-Starke, B., Beylich, A., Moser, T., Nikolakis, A., Rumpler, N., Schäffer, A., et al. (2013). The response of soil organism communities to the application of the insecticide lindane in terrestrial model ecosystems. *Ecotoxicology* 22, 339–362. doi: 10.1007/s10646-012-1030-0

Schouten, A. J., Breure, A. M., Bloem, J., Didden, W., De Ruiter, P. C., and Siepel, H. (1999). *Life Support Functions of the Soil: Operationalization for the Policy*. RIVM Report 607601003, National Institute of Public health and the Environment, Bilthoven.

Schrader, S., Thiele, J.-A., and Pacholski, A. (2005). Bodenökologische Bewertung eines Agrarökosystems anhand der räumlichen Variabilität ausgewählter Parameter. *Mitteilungen der Deutschen Bodenkundlichen Gesellschaft* 107, 205–206.

Scoriza, R. N., Silva, A. D., Correia, M. E. F., Leles, P. S. D., and de Resende, A. S. (2015). Herbicide use in degraded forest areas in restoration: effects on soil invertebrate biota. *Rev. Brasil. Ciencia Solo* 39, 1576–1584. doi: 10.1590/01000683rbcs20150096

Scott-Fordsmand, J. J., Maraldo, K., and Van den Brink, P. (2008). The toxicity of copper contaminated soil using a gnotobiotic Soil Multi-species test System (SMS). *Environ. Int.* 34, 524–530. doi: 10.1016/j.envint.2007.11.008

Sechi, V., D'Annibale, A., Maraldo, K., Johansen, A., and Bossi, R. J. (2014). Species composition of a soil invertebrate multi-species test system determines the level of ecotoxicity. *Environ. Pollut.* 184, 586–596. doi: 10.1016/j.envpol.2013.10.008

Silva, A. L. P., Amorim, M. J. B., and Holmstrup, M. (2015). Salinity changes impact of hazardous chemicals in *Enchytraeus albidus*. *Environ. Toxicol. Chem.* 34, 2159–2166. doi: 10.1002/etc.3058

Silva, A. L. P., Amorim, M. J. B., and Holmstrup, M. (2016). Adaptations of enchytraeids to single and combined effects of physical and chemical stressors. *Environ. Rev.* 24, 1–12. doi: 10.1139/er-2015-0048

Spurgeon, D. J., Morgan, A. J., and Kille, P. (2008). "Current research in soil invertebrate ecotoxicogenomics," in *Comparative Toxicogenomics,* eds C. Hogstrand and P. Kille (Oxford: Elsevier), 133–163.

Standen, V. (1973). The production and respiration of an enchytraeid population in blanked bog. *J. Animal Ecol.* 42, 219–245. doi: 10.2307/3282

Standen, V. (1984). Production and diversity of enchytraeid earthworms and plants in fertilized hay meadow plots. *J. Animal Ecol.* 21, 293–312. doi: 10.2307/2403055

Stringer, A., and Wright, M. A. (1973). The effect of benomyl and some Related compounds on *Lumbricus terrestris* and other earthworms. *Pest. Sci.* 4, 165–170. doi: 10.1002/ps.2780040202

Topoliantz, S., Ponge, J.-F., and Viaux, P. (2000). Earthworm and enchytraeid activity under different arable farming systems, as exemplified by biogenic structures. *Plant Soil* 225, 39–51. doi: 10.1023/A:1026537632468

Turbé, A., De Toni, A., Benito, P., Lavelle, P., Ruiz, N., Van der Putten, W. H., et al. (2010). *Soil Biodiversity: Functions, Threats, and Tools for Policy Makers. - BioIntelligence Service. IRD. and NIOO.* Report for European Commission (DG Environment), Brussels.

Van den Brande, J., and Heungens, A. (1969). Influence of repeated applications of nematicides on the soil fauna in Begonia culture. *Neth. J. Plant Pathol.* 75, 40–44. doi: 10.1007/BF02137191

Van Gestel, C. A. M. (2012). Soil ecotoxicology: state of the art and future directions. *ZooKeys* 176, 275–296. doi: 10.3897/zookeys.176.2275

Van Vliet, P. C. J., West, L. T., Hendrix, P. F., and Coleman, D. C. (1993). The influence of Enchytraeidae (Oligochaeta) on the soil porosity of small microcosms. *Geoderma* 56, 287–299. doi: 10.1016/0016-7061(93)90118-5

VICH (2005). *Environmental Impact Assessment (EIAs) for Veterinary Medicinal Products (VMPs) – Phase II Guidance. VICH Guideline 38 (Ecotoxicity Phase II).* CVMP/VICH/790/03-FINAL. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, London.

Voronova, L. D. (1968). The effect of some pesticides on the soil invertebrate fauna in the south taiga zone in the Perm region (USSR). *Pedobiologia* 8, 507–525.

Voua Otomo, P., Wahl, J., and Maboeta, M. S. (2013). The enchytraeid reproduction test (ERT): a potentially quick and affordable tool for the

assessment of metal contaminated soils in emerging economies. *Bull. Environ. Contam. Toxicol.* 91, 545–548. doi: 10.1007/s00128-013-1092-6

Waichman, A. V., Römbke, J., Ribeiro, M. O. A., and Nina, N. C. S. (2002). Use and fate of pesticides in the Amazon State, Brazil. Risk to human health and the environment. *Environ. Sci. Pollut. Res.* 9, 423–428. doi: 10.1007/BF02987596

Way, M. Y., and Scopes, N. E. A. (1968). Studies on the persistence and effects on soil fauna of some soil applied systemic insecticides. *Ann. Appl. Biol.* 62, 199–214. doi: 10.1111/j.1744-7348.1968.tb02816.x

Weber, G. (1953). Die Makrofauna leichter und schwerer Ackerböden und ihre Beein-flussung durch Pflanzenschutzmittel. *Zeitschrift Pflanzenernährung Düngung Bodenkunde* 61, 107–118. doi: 10.1002/jpln.19530610203

Westheide, W., Bethke Beilfuß, D., and Gebbe, J. (1991). Effects of Benomyl on reproduction and population structure of enchytraeid oligochaetes (Annelida). Sublethal tests on agar and soil. *Comp. Biochem. Physiol.* 100C, 221–224. doi: 10.1016/0742-8413(91)90157-o

Weuffen, W. (1968). Zusammenhänge zwischen chemischer Konstitution und keimwidriger Wirkung. *20. Archiv Experimentelle Veterinärmedizin* 22, 127–132.

Weyers, A., Römbke, J., Moser, T., and Ratte, H.-T. (2002). Statistical results and implications of the Enchytraeid Reproduction Test. *Environ. Sci. Technol.* 36, 2116–2121. doi: 10.1021/es000259h

Weyers, A., Sokull-Klüttgen, B., Knacker, T., Martin, S., and Van Gestel, C. A. M. (2004). Use of Terrestrial Model Ecosystem data in environmental risk assessment for industrial chemicals, biocides and plant protection products in the EU. *Ecotoxicology* 13, 163–176. doi: 10.1023/B:ECTX.0000012412.44625.69

Yang, D., Zhu, J., Fu, R., Wang, W., Guo, X., Wang, Z., et al. (2012a). Enchytraeidae *Fridericia bulbosa* as a new test species for soil ecotoxicity assessment. *Chemosphere* 88, 501–506. doi: 10.1016/j.chemosphere.2012.03.007

Yang, D., Zhu, J., Shen, G. X., Wang, W. H., Guo, X. P., Wang, Z. Q., et al. (2012b). The acute toxicity of single and combined exposure of mercury and bromoxynil on *Fridericia bulbosa*. *Appl. Mech. Mater.* 137, 280–285. doi: 10.4028/www.scientific.net/AMM.137.280

Zhao, S., Fung-Leung, W. P., Bittner, A., Ngo, K., and Liu, X. (2014). Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. *PLoS ONE* 9:e78644. doi: 10.1371/journal.pone.0078644

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Römbke, Schmelz and Pélosi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Environmental Science

REVIEW
published: 25 September 2018
doi: 10.3389/fenvs.2018.00105

Aquatic Fungi: A Disregarded Trophic Level in Ecological Risk Assessment of Organic Fungicides

Lukas D. Ittner†, Marion Junghans† and Inge Werner*

Swiss Centre for Applied Ecotoxicology Eawag, École Polytechnique Fédérale de Lausanne (EPFL), Dübendorf, Switzerland

Freshwater fungi are a diverse group of organisms and fulfill important functions in the food web dynamics of surface water ecosystems. Ascomycetic and basidiomycetic hyphomycetes play key roles in leaf litter breakdown in rivers and creeks, while parasitic chytrids are an important food source for small invertebrates in lakes. Field studies indicate that fungal communities are affected by fungicides at environmentally relevant concentrations. However, despite their ecological importance, freshwater fungi are currently not specifically addressed in the EU regulatory frameworks with respect to the protection of surface waters. Specifically, the prospective risk assessment of fungicides does not evaluate adverse effects on non-target aquatic fungi. This paper aims to describe important functions of freshwater fungi, provides an overview of adverse effect levels of fungicides on this organism group, and proposes to integrate the fungal community of freshwater ecosystems as an additional trophic level in the current fungicide risk assessment frameworks. Results of a literature review on the effects of fungicides on aquatic fungi revealed that information on the toxicity of fungicides to non-target aquatic fungi is limited. This is, in part, due to the lack of standardized bioassays using aquatic fungi as test species. Although there is an encouraging number of bioassays focusing on the degradation of dead organic material by hyphomycetes, studies on fungicide effects on other important ecological functions, like the control of algal blooms in lentic surface waters by parasitic chytrid fungi, or on mutualistic fungi living in the guts of aquatic arthropods are largely missing. Thus, the further development and standardized of different fungi bioassays is recommended.

Keywords: fungal ecology, fungal diversity, plant protection products, biocides, water framework directive, policy analysis

OPEN ACCESS

Edited by:
Carsten A. Brühl,
Universität Koblenz Landau, Germany

Reviewed by:
Yong Liu,
Hunan Academy of Agricultural Sciences (CAAS), China
Fernando José Cebola Lidon,
Universidade Nova de Lisboa, Portugal
Joan Artigas,
UMR6023 Laboratoire Microorganismes Génome Et Environnement (LMGE), France

***Correspondence:**
Marion Junghans
Marion.Junghans@oekotoxzentrum.ch

†These authors share first authorship

Specialty section:
This article was submitted to Agroecology and Ecosystem Services, a section of the journal Frontiers in Environmental Science

Received: *31 March 2017*
Accepted: *27 August 2018*
Published: *25 September 2018*

Citation:
Ittner LD, Junghans M and Werner I (2018) Aquatic Fungi: A Disregarded Trophic Level in Ecological Risk Assessment of Organic Fungicides. Front. Environ. Sci. 6:105. doi: 10.3389/fenvs.2018.00105

BACKGROUND

One of the most important anthropogenic hazards for the ecological health of freshwater ecosystems is the input of pesticides (biocides and plant production products) via point sources such as wastewater treatment plants (mainly biocides) as well as non-point sources, such as spray drift, drainage and run-off from agricultural fields (e.g., Petersen et al., 2013; Moschet et al., 2014). To protect the ecology of water bodies from adverse effects of plant protection products (PPP), a prospective risk assessment is conducted by the European Food Safety Authority (EFSA) prior to authorization of active ingredients and their formulated products. The EFSA guidance document (EFSA, 2013), requires toxicity data for three taxonomic groups: plants (e.g., algae, duckweed),

invertebrates (e.g., cladoceran crustacea e.g., *Daphnia magna*) and a fish species, representing a simplified food chain consisting of primary producers, primary consumers, and secondary consumers. Similar approaches are used for the authorization of biocides (European Chemicals Agency, 2015) as well as for deriving environmental quality standards (EQS) for retrospective risk assessment under the EU Water Framework Directive (WFD, EU 2000).

The most recent version of the EFSA guidance document (EFSA, 2013) acknowledges that studies by Maltby et al. (2009); Bundschuh et al. (2011); Dijksterhuis et al. (2011), and Zubrod et al. (2015a) give reason for concern that the current data requirements for ecological risk assessment does not adequately consider the risk of fungicides for aquatic fungi. In addition, recent studies suggest that aquatic fungi are particularly sensitive to ergosterol-inhibiting fungicides such as triazoles [Dijksterhuis et al. (2011), Dimitrov et al. (2014), Zubrod et al. (2015b) and references therein].

Freshwater fungi are a diverse group of organisms and fulfill important functions in the food web dynamics of surface water ecosystems. They play a key role in the breakdown of allochthonous (foreign to a certain environment) organic material such as twigs, leaves, etc. which provides up to 99% of the total energy input into surface waters (Teal, 1957; Nelson and Scott, 1962; Fisher and Likens, 1973; Bärlocher and Kendrick, 1974). The colonization of organic material by microorganisms and aquatic fungi therefore represents an essential component of the food web of running waters. Due to the large diversity of fungi as well as the scarcity of toxicity data for relevant fungal species EFSA identified the development of standardized ecotoxicity assays as a future research need (EFSA, 2013). Such data are also needed for the derivation of EQS for fungicides under the WFD, which aim at protecting the most sensitive taxonomic groups. Without data on the sensitivity of aquatic fungi, higher assessment factors have to be applied[1]. An overview on considering aquatic fungi in fungicide risk assessment under different regulatory frameworks can be found in **Supplementary Data Sheet 1**. It shows that fungal bioassays focussing on ecosystem functioning as well as on community structure are needed.

This paper provides an overview on the current classification and ecology of fungi in freshwater ecosystems, addresses fungicide exposure in surface waters, and reviews current information on the effects of organic fungicides on freshwater fungi. Inorganic fungicides such as copper were not considered. Information on the effect of copper and other heavy metals can be found elsewhere (e.g., Duddridge and Wainwright, 1980; Jaeckel et al., 2005b; Pascoal et al., 2005; Azevedo et al., 2007; Roussel et al., 2008; Solé et al., 2008; Sridhar et al., 2008; Zubrod et al., 2015a). Furthermore, relevant taxonomic groups are recommended for bioassay development or improvement.

[1]For deriving the environmental quality standard accounting for acute ecotoxicity, the maximum acceptable concentration environmental quality standard (MAC-EQS) for fungicides, the availability of fungi EC50 values is needed to lower the standard AF from 100 to 10 (European Commission, 2011): "For substances with a specific mode of action the most sensitive taxa can be predicted with confidence. Where representatives of the most sensitive taxa are present in the acute dataset, an AF < 100 may again be justified".

BIODIVERSITY OF FUNGI IN FRESHWATER ECOSYSTEMS

Within the domain Eukaryota, fungi represent their own kingdom (**Figure 1**) and are hence on the same taxonomic level as animals, plants and protists (Woese and Fox, 1977; Woese et al., 1990). Over the last few decades, the taxonomy of fungi has changed considerably as a consequence of genetic analyses (Voigt and Kirk, 2011), and a fungal tree of life was generated by Lutzoni et al. (2004), James et al. (2006), and Hibbett et al. (2007), whose taxonomy is used in this paper.

The total number of fungal species is estimated at 1.5 Million (Hawksworth 1991, 2001), while only approximately 7% of these species have been described (Mueller and Schmit, 2007). About 3,000 fungal species and 138 non-fungal oomycetes have been reported to be present in aquatic habitats. The greatest biodiversity of these groups was described for temperate areas (Shearer et al., 2007). Goh and Hyde (1996) reported over 600 freshwater species, consisting of ca. 300 ascomycetes, 300 mitosporic fungi, and a number of chytridiomycetes and non-fungal oomycetes. It can be assumed that just a small fraction of the aquatic fungal community has been described so far and that the number of newly discovered species will increase rapidly (Goh and Hyde, 1996; Shearer et al., 2007; Voigt and Kirk, 2011).

Various classifications of freshwater fungi exist (Goh and Hyde, 1996; Wong et al., 1998; Shearer et al., 2007). Most of the species living in freshwater habitats have been ascribed to the phyla ascomycetes, basidiomycetes, chytridiomycetes, and glomeromycetes (Shearer et al., 2007). The latter includes the zygomycetes, which formerly formed their own phylum (Hibbett et al., 2007). Wurzbacher et al. (2010) and Krauss et al. (2011) used a classification that focuses more on their functional traits in freshwater ecosystems rather than on phylogeny. Since this focus is beneficial for characterizing the effects of fungicides in freshwater ecosystems, their classification is adopted for this review. They proposed the following four main groups: (1) aquatic hyphomycetes (also called freshwater hyphomycetes or Ingoldian fungi), (2) chytridiomycetes (also called chytrids), (3) yeasts, and (4) glomeromycetes. While the majority of these groups can be regarded as being monophyletic, the aquatic hyphomycetes mainly belong to the ascomycetes with a small proportion in the basidiomycetes. Also, yeasts represent a polyphyletic group consisting of ascomycetes and basidiomycetes (Shearer et al., 2007). The differentiation between hyphomycetes and yeasts is hence mainly determined by their different morphology. The oomycetes (5) are treated as an additional but separate group (Shearer et al., 2007), since they are non-fungal from a taxonomical point of view. Their consideration for this review nonetheless is reasonable because they occupy similar niches as aquatic fungi and fulfill fungal-like ecological functions in freshwater ecosystems (Wong et al., 1998). The five functional fungal or fungal-like groups are described in more detail below:

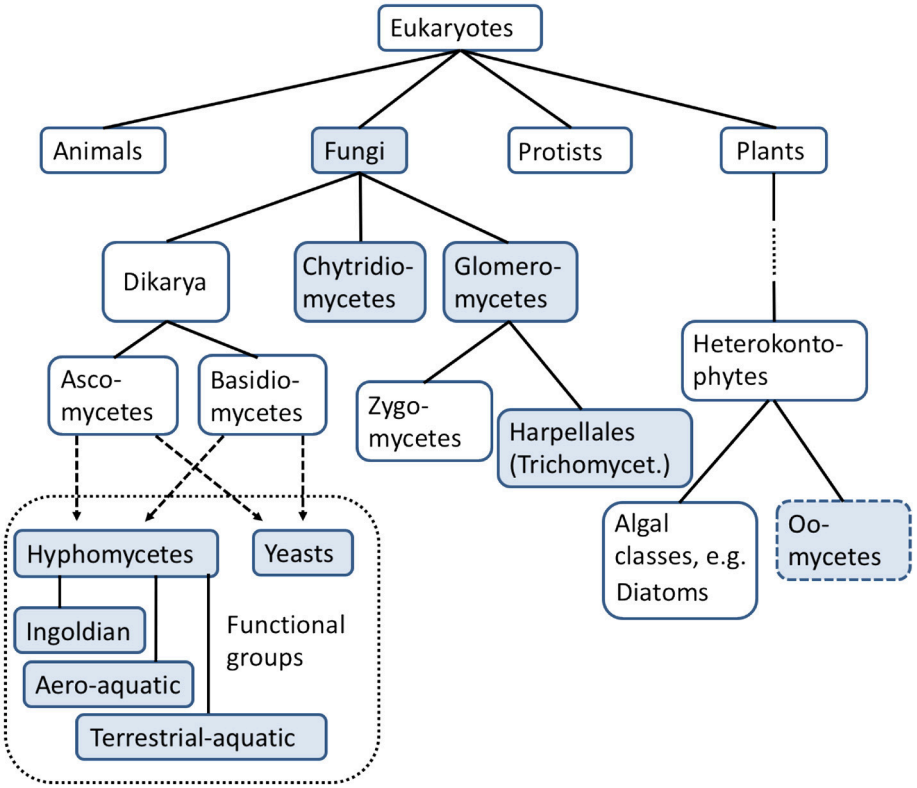


FIGURE 1 | Taxonomic position of aquatic fungi in relation to current standard test organisms (fungal taxonomy based on Hibbett et al., 2007; Wurzbacher et al., 2010; Krauss et al., 2011). Taxonomic groups with filled boxes are subject of this review.

(1) Aquatic hyphomycetes probably represent the most well-studied group and are reported to be part of freshwater ecosystems all over the world (Wong et al., 1998). Traditionally, they are distinguished into two groups based on their biological behavior (Goh and Hyde, 1996): (i) the Ingoldian fungi which are characterized by their ability to sporulate under water, and (ii) the aero-aquatic fungi which do not accomplish their whole life cycle under water, needing air exposure for reproduction (Wurzbacher et al., 2010). Goh and Hyde (1996, and references therein), further discern the (iii) submerged-aquatic hyphomycetes which are regarded as "facultative-aquatic," since they do not sporulate primarily under water. All these hyphomycete groups are commonly found on submerged plant material (e.g., leaves, twigs, wood, etc.). Finally, there are also terrestrial-aquatic hyphomycetes, (e.g., occurring in rain drops associated with intact terrestrial plant material such as leaf surfaces) but since their habitat is outside aquatic ecosystems they are not considered any further.

(2) The chytridiomycetes are also a well-documented group (Wong et al., 1998), but little is known about their ecology (Gleason et al., 2008). They commonly are parasitic or saprotrophic and typically occur in the pelagic zone of stagnant waters (Wurzbacher et al., 2010).

(3) Yeasts are a ubiquitous fungal-group found virtually everywhere in freshwater ecosystems, especially in the pelagic zone of lakes (Wurzbacher et al., 2010). Despite several studies on aquatic yeasts the knowledge about their ecology is generally limited (Ahearn et al., 1968; Wurzbacher et al., 2010), and there exists no comprehensive analysis on yeast ecology and their role in freshwater ecosystems.

(4) The glomeromycetes also represent a group for which little is known regarding their occurrence and ecology in freshwater environments (Goh and Hyde, 1996). Most species of this group are terrestrial (Shearer et al., 2007). An exception are the trichomycetes which live parasitically or mutualistically (mutual advantages for both partners) in the digestive tract of aquatic arthropods (Shearer et al., 2007; Hernández Roa et al., 2009; Jobard et al., 2010). The trichomycetes are considered to be a polyphyletic group (Hibbett et al., 2007), partially belonging to the protists (Benny and O'Donnell, 2000; Cafaro, 2005). The trichomycete order harpellales, for which mutualistic species have been reported (Jobard et al., 2010), is considered to belong to the glomeromycetes (Hibbett et al., 2007).

(5) The non-fungal oomycetes are well-documented (Wong et al., 1998) and among the most ubiquitous aquatic microbes on earth (Shearer et al., 2007). The majority of species in this group lives saprotrophically, whereas some of them are animal parasites (e.g., on fish and crustaceans) or plant pathogens (Shearer et al., 2007). New research suggests that oomycetes are taxonomically related to certain algae such as phaeophytes (brown algae) or

bacillariophytes (diatoms), showing their close affiliation with plants (Adl et al., 2005). According to (Voigt and Kirk, 2011) oomycetes are algae without chloroplasts but with cellulose in their cell walls (**Figure 1**).

Currently, different ways exist to identify aquatic fungi to the species level. For instance, Lin et al. (2012) identified aquatic fungi via the conidial morphology. A more innovative and future-oriented identification method is the determination by means of genetic studies, since results are more reliable and accurate (Krauss et al., 2011). Also, community fingerprinting techniques have proven useful to study the fungal diversity in microcosms [Krauss et al. (2011) and references therein].

IMPORTANT ROLES OF FUNGI AND OOMYCETES IN FRESHWATER ECOSYSTEMS

Degradation of Dead Organic Material

A key function of aquatic fungi is the degradation of dead plant or other organic material (e.g., chitin, keratin; **Figure 2**). The decomposition of so called "standing-dead" emergent plants and submerged terrestrial plant litter (primarily leaves) by aquatic hyphomycetes in lentic and lotic waters respectively, plays a substantial role (Gessner et al., 2007) in the nutrient cycle of aquatic systems. While aero-aquatic fungi and yeasts predominantly occur on plant material of stagnant waters, ditches or slow-flowing streams under low to semi-aerobic conditions, the Ingoldian fungi are usually found in great numbers on submerged plant material (primarily leaves and twigs) in fast-flowing tree-lined streams and brooks and well-aerated lakes (Goh and Hyde, 1996; Wurzbacher et al., 2010). The submerged aquatic hyphomycetes prefer similar habitat conditions to the Ingoldian fungi, but are mostly detected on woody material (Goh and Hyde, 1996).

The degradation of dead plant material results in the production of fungal biomass, the formation of reproductive spores, litter transformation products as dissolved organic matter (DOM) and fine particulate organic matter (FPOM). This process also increases the food quality for shredders (Cummins, 1974; Wong et al., 1998; Gessner et al., 1999, 2007) and food availability for other aquatic invertebrates. Since the input of allochthonous organic material (e.g., leaves, twigs, wood etc.) is considered the main energy source in low order forested streams (Teal, 1957; Nelson and Scott, 1962; Fisher and Likens, 1973; Bärlocher and Kendrick, 1974; Cummins, 1974)–exceeding the primary production in those waters—the degradation of dead plant material by aquatic fungi can be regarded as a critical component in the food web dynamics of these freshwater ecosystems. In addition, pollen and non-plant material is degraded mainly by chytridiomycetes and the non-fungal oomycetes (Goh and Hyde, 1996; Shearer et al., 2007; Gleason et al., 2008; Kagami et al., 2014; Wurzbacher et al., 2014), resulting in biomass and spores that can also be used as a food source by invertebrates.

Parasitism and Mutualism

The role of aquatic fungi (especially chytridiomycetes) and oomycetes as parasites in freshwater ecosystems is currently poorly understood. Fungal parasitism can greatly influence food supply, nutrient transfer and population dynamics in freshwater ecosystems (Kagami, 2008; Miki et al., 2011). Though parasitism is often not clearly distinguishable from mutualism (Jobard et al., 2010), there is evidence that both parasitic and mutualistic fungal species exist (Lichtwardt and Williams, 1999; Shearer et al., 2007; Strongman, 2007; Hernández Roa et al., 2009; Jobard et al., 2010). Examples of such mutualistic and/or parasitic fungi are the trichomycetes. They belong to the glomeromycetes and live in the guts of insects, crustaceans and millipedes (Fisher and Likens, 1973; Lichtwardt and Williams, 1999; Strongman, 2007). The knowledge about trichomycetes is scarce and thus their role and importance in food webs of aquatic ecosystems is still unclear (Jobard et al., 2010).

One of the most significant parasitism-host interactions is the association of parasitic chytridiomycetes with phytoplankton. On the one hand, chytridiomycetes, can serve as an important high-quality food source (polyunsaturated fatty acids, cholesterol) for zooplankton (e.g., daphnids) via biomass production (e.g., formation of zoospores; Müller-Navarra et al., 2000; Kagami et al., 2007; Miki et al., 2011). On the other hand, these fungi can control phytoplankton seasonal succession (Kagami et al., 2007; Miki et al., 2011), thereby preventing algal blooms. This demonstrates the importance of parasitic aquatic fungi in influencing population dynamics. Chytridiomycetes also represent a direct link between sinking, oversized, and hence non-accessible phytoplankton and filter-feeding zooplankton such as daphnids in the pelagic zone (Kagami et al., 2007; Jobard et al., 2010; Wurzbacher et al., 2010; Miki et al., 2011; Rasconi et al., 2014). Based on Kagami et al. (2007) and Kagami et al. (2014), this nutrient transfer between different trophic levels was termed "mycoloop," and it underlines the significance of parasitic fungi as a crucial factor in food web dynamics of freshwater ecosystems.

Sequestration and Degradation of Xenobiotics and Nutrient Dynamics

From an ecotoxicological perspective, aquatic fungi can be important for the sequestration of heavy metal ions (e.g., cadmium, copper, zinc, lead) and the breakdown of organic xenobiotic compounds (e.g., nonylphenol, bisphenol A, 1-naphtol) in freshwater ecosystems (Jaeckel et al., 2005a; Augustin et al., 2006; Azevedo et al., 2007; Wurzbacher et al., 2010; Bärlocher et al., 2011; Krauss et al., 2011; Omoike et al., 2013; Lucas et al., 2016; Martínková et al., 2016; Oliveira et al., 2016). For example, aquatic fungi can sequester greater amounts of heavy metals than bacteria (Massaccesi et al., 2002), and outweigh bacteria in biomass (Findlay and Arsuffi, 1989). Recent studies showed that some fungi are able to degrade herbicides, insecticides (Oliveira et al., 2015) and even fungicides (Inoue et al., 2015). The ability to degrade and detoxify organic as well as inorganic pollutants suggests that aquatic fungi could play a role in the improvement of water quality and in biotechnological applications.

Fungi associated with decaying plant material (mainly aquatic hyphomycetes) directly influence the nutrient dynamics of

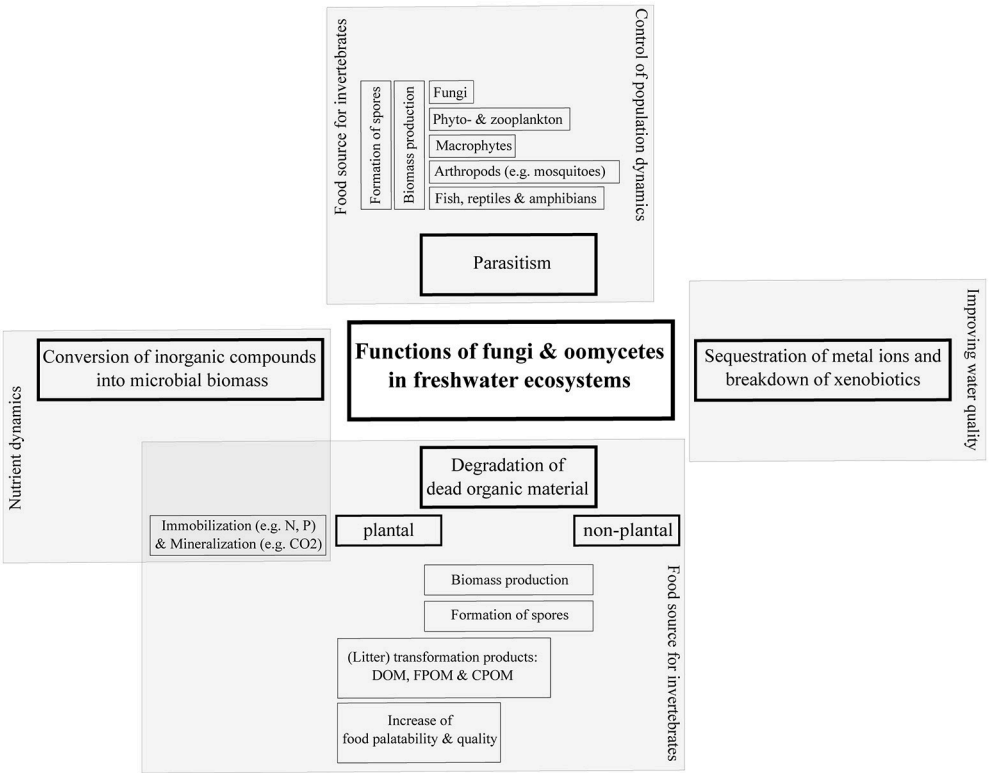


FIGURE 2 | Direct and indirect functions of aquatic fungi and oomycetes in freshwater ecosystems. DOM, dissolved organic matter; FPOM, fine particulate organic matter; CPOM, coarse particulate organic matter.

freshwater ecosystems by mineralization of organic carbon to carbon dioxide (CO_2) as well as by conversion of inorganic compounds, e.g., nitrogen (N) and phosphorus (P), into microbial biomass. For instance, chytridiomycetes are able to convert inorganic nitrogen, inorganic sulfur and inorganic phosphorus to organic compounds, which then can become available to heterotrophic organisms in ecosystems [Gleason et al. (2008) and references therein].

EXPOSURE AND EFFECTS OF FUNGICIDES ON FRESHWATER FUNGI

Fungicides primarily enter surface waters via non-point sources such as agricultural runoff (e.g., Cruzeiro et al., 2015). Concentrations of fungicides in surface waters therefore fluctuate during the growing season, showing strong temporal and spatial variability (e.g., Rabiet et al., 2010; Bereswil et al., 2012; Moschet et al., 2014; Spycher et al., 2018). Two monitoring studies performed in 2012 in Switzerland and Norway provide insight into fungicide contamination in agriculturally influenced catchments over the course of an entire growing season. Moschet et al. (2014) showed that next to herbicides, fungicides were the second most abundant pesticides detected in medium sized rivers flowing through agricultural areas. Of 13 fungicides that were detected in at least three of five rivers, 7 (azoxystrobin, cyproconazole, carbendazim, tebuconazole, dimethomorph, propamocarb and metalaxyl-M) were detected in >70% of the samples analyzed, with maximum concentrations ranging from 18 ng/l (fenamidone) to 380 ng/l (metalaxyl-M). Petersen et al. (2013) detected at least one fungicide in 57% and >4 fungicides in 9% of 54 water samples. Detection frequency was highest in areas of potato and vegetable production (78%). Maximum fungicide concentrations ranged from 37 ng/l (imazalil) to 680 ng/l (fenamidone). In both studies, herbicides dominated in terms of detected active substances (77 and 58% of samples, respectively), however, the abundance of fungicides can equal or exceeded that of herbicides in catchments with a higher share of orchards and vineyards. This was shown by Kreuger et al. (2010) and Spycher et al. (2018). The latter measured a peak concentration of 6 µg/L fluopyram in a 0.5 day composite sample collected from a stream situated in a wine growing area near the shore of Lake Geneva. There, fungicides accounted for 64% of pesticides detected. Compared to herbicides which tend to be relatively water soluble, fungicides are rather lipophilic. The average octanol-water partition coefficient (logKow) for 45 fungicides detected by Spycher et al. (2018) is 3.4 (5th percentile = 1.8; 95th percentile = 4.7). The frequent occurrence of fungicides in agriculturally influenced streams along with their tendency to bind to organic matter suggest that aquatic fungi, especially leaf litter associated hyphomycetes,

are exposed to fungicides both via the water phase and their substrate.

Fungicides detected in the studies described above belong to a wide variety of chemical classes with different modes of action, e.g., anilinopyrimides (inhibition of aminoacid synthesis), azoles (inhibition of sterol synthesis), benzimidazoles (inhibition of beta tubulin synthesis), carbamates (inhibition of phospholipid and fatty acid synthesis), carboxylic acid amides (inhibition of cell wall biosynthesis), pyridine-carboxamides (respiration), phenylamides (nucleic acid synthesis), and strobilurins (inhibition of mitochondrial respiration). They comprise almost all classes listed on the Fungicide Resistance Action Committee website (www.frac.info), which provides a comprehensive overview on fungicidal modes of action. A similar spectrum of fungicide classes was detected in a 2013 study on pesticide exposure in 100 streams in agricultural and urban areas of the midwestern United States (Van Metre et al., 2017; Nowell et al., 2018). The authors analyzed extracts of POCIS passive samplers in addition to water samples. Results of the study show that strobilurins such as azoxystrobin, azoles such as tebuconazole as well as benzimidazoles such as carbendazim are of relevance world-wide, and highlight that fungicide pollution might be of similar or even higher importance in urban catchments.

Although adverse effects of organic fungicides on non-target aquatic fungi might be expected and their widespread application in agriculture (Sungur and Tunur, 2012), little information exists both for active fungicidal substances and formulated products. The literature available on this topic is described below. Some authors studied effects on fungal species abundance (i.e., structural endpoints) as well as functional endpoints (**Table 1**), whereas others only focused on leaf litter breakdown as a functional endpoint (**Table 2**).

Field studies indicate that fungicides affect microbial communities at environmentally relevant concentrations. Wilson et al. (2014) have found that guts of black fly larvae were less infested with mutualistic trychomycetes in agriculturally influenced streams. Fernández et al. (2015) found a correlation between structural changes in microbial communities as well as fungal biomass with increasing predicted fungicide toxicity based on the chemical analysis and toxic unit calculation by combining fungicide monitoring with field studies on fungal communities. Rossi et al. (2017) found differences in fungi community structure between alder leaves exposed in a pristine part of a stream and those exposed at a downstream site where several fungicides were detected by chemical analysis. Gardeström et al. (2016) observed that fungal communities from an agriculturally influenced stream were more tolerant to azoxystrobin than a community without a history of pesticide exposure, indicating a shift in community composition toward tolerant species, also known as pollution induced community tolerance (Molander et al., 1990). The observations from these studies stress the need for considering the hazard to aquatic fungi in fungicide risk assessment, and for new toxicity tests with integral endpoints.

Several studies on structural endpoints analyzed the effects of organic fungicides on fungal communities collected from submerged leaf litter either exposed on leaves, or on agar plates. Bärlocher and Premdas (1988) analyzed the effects of pentachlorophenol (PCP), a non-selective PPP with general biocidal activity (Tomlin, 2009), on aquatic hyphomycetes (**Table 1**). They found evidence for reduced reproduction (conidia count) and metabolic stress (increased respiration of microbial community on leaf disks) at PCP concentrations of 1 to 1,000 μg/l, with a peak increase at 100 μg/l. Chandrashekar and Kaveriappa (1989) studied the effects of mancozeb and captafol on the growth of aquatic hyphomycetes (**Table 1**). Both caused no growth inhibition in three fungal species up to a concentration of 5 mg/l, while total inhibition of growth was observed at 500 mg/l to 1000 mg/l. Later Chandrashekar and Kaveriappa (1994) examined the impact of mancozeb, captafol, carbendazim, tridemorph on conidia sporulation and germination in different aquatic hyphomycetes species (**Table 1**). None of the tested fungicides or other pesticides had inhibitory effects on sporulation or germination at concentrations of $\leq$5 mg/l and $\leq$1 mg/l, respectively. Mancozeb, tridemorph, and carbendazim inhibited sporulation of all test species at 500 mg/l and captafol at 2500 mg/l. Conidia germination was inhibited at 1,000 mg/l captafol and 1,000 mg/l mancozeb.

Dijksterhuis et al. (2011) were the first to include species from fungal groups (yeasts, glomeromycetes) and non-fungal groups (oomycetes) other than the aquatic hyphomycetes. They tested the effects of carbendazim, chlorothalonil, fluazinam, imazalil, epoxiconazole, tebuconazole, and azoxystrobin on 6 non-target aquatic fungal species and non-fungal oomycetes isolated from the environment. The authors observed that a comprehensive protection of aquatic fungi and oomycetes in freshwater ecosystems may not be guaranteed through the currently applied standard risk assessment for aquatic organisms for the two tested triazoles, epoxiconazole and tebuconazole, as well as for azoxystrobin. The oomycetes were the most sensitive group for azoxystrobin. Four out of the 6 fungal species (*Cryptococcus flavescens*, *Trichoderma hamatum*, *Fusarium sporotrichioides*, *Mucor hiemalis*) showed high sensitivity to triazoles, whose mode of action is the inhibition of sterol biosysnthesis. The NOEC for the triazole tebuconazole was lower than an HC5 value derived by Maltby et al. (2009) for these substances with SSDs generated from data on non-fungal species NOECs. A similar finding is reported in Dimitrov et al. (2014), who studied tebuconazole in a lentic water system. The tested concentration of 238 μg/l represents the HC5 of the SSD constructed with acute EC50 values for fish invertebrates and primary producers. While no significant effects were observed for leaf litter decomposition or fungal biomass, a significant reduction in conidia production as well as change in the fungal community composition was observed. Donnadieu et al. (2016) observed a negative effect on fungal biomass after exposure to a single, environmentally relevant concentration of tebuconazole (10.7 μg/l) in indoor streams. Concurrently, bacterial biomass increased. Additionally, the spore number indicated a significant shift in ascomycete composition. The authors concluded that a risk assessment for azole fungicides that is based on vertebrates, invertebrates and primary producers alone may not be protective for the structure and functioning of freshwater ecosystems.

TABLE 1 | Summary of literature on the effects of fungicides on aquatic fungi in freshwater.

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal species	Tested fungal taxa	Lowest toxicity value
Bärlocher and Premdas, 1988	Pentachloro-phenol	(1)	Sporulation of conidia, respiration	Maple leaves that were pre-conditioned for 1-2 months in a brook: (i) conidia harvested from the leaves after 48h exposure, (ii) oxygen consumption by microbial communities measured on intact leaves	***Aquatic hyphomycetes:*** *Clavariopsis aquatica* (de Wild), *Articulospora tetracladia* (Ingold), *Alatospora aquatica* (Ingold)	Not specified	No NOEC can be derived but authors wrote: the 0.1 μg/L treatment does not significantly differ from the control at 10 000 μg/L conidia "often showed structural abnormalities" Figure 1 suggests that conidia production was decreased at 100, 1000 and 10000 μg/L
Chandrashekar and Kaveriappa, 1989	Mancozeb (as Dithane M-45)	(2)	Growth (biomass)	*Hyphomycetes* isolated from submerged leaf litter and maintained on agar, mycelial discs cut from 10-day old agar plate cultures placed in treated medium, determination of radial growth after incubation at room temperature for 10 days	***Aquatic hyphomycetes:*** *Flagellospora penicillioides*, *Lunulospora curvula*, *Phalangispora constricta*	*Ascomycetes*, *oomycetes*	EC50: *F. penicillioides*: 350 mg/L *L. curvula*: 350 mg/L *P. constricta*: 500 mg/L
	Captafol (as Foltap)	(3)				*Ascomycetes*, *oomycetes*	EC50: *F. penicillioides*: 350 mg/L *L. curvula*: 350 mg/L 500 mg/L *(P. constricta)*
Chandrashekar and Kaveriappa, 1994	Mancozeb (as Dithane M-45)	(2)	Sporulation and germination of conidia	Coffee and rubber leaves collected from a free-flowing stream, Sporulation: leaves cut into pieces and incubated for 60 days at room temperature in test medium, observation of conidia of different *hyphomycetes* species; Germination: leaf pieces incubated in distilled water for 24-48 h, harvesting of conidia, exposure to test concentrations in cavity slides for 24h, counting of germinated conidia under the microscope	***Aquatic hyphomycetes:*** *Anguillospora crassa*, *Anguillospora longissima*, *Anguillospora spp*, *Beltrania rhombica*, *Campylospora chaetocladia*, *Campylospora filicladia*, *Flabellospora crassa*, *Flabellospora verticillata*, *Flagellospora penicilliodes*, *Helicosporium spp*, *Lunulospora curvula*, *Lunulospora cymbiformes*, *Phalangispora constricta*, *Triscelophorus acuminatus*, *Triscelophorus monosporus*, *Triscelophorus spp*, *Wiesneriomyces javanicus*	*Ascomycetes*, *oomycetes*	NOEC (conidia germination): 1 mg/L
	Captafol (as Foltap)	(3)				*Ascomycetes*, *oomycetes*	NOEC (conidia germination): 1 mg/L
	Tridemorph (as Calixin)	(4)				*Ascomycetes*, *basidiomycetes*	NOEC (conidia germination): 1 mg/L
	Carbendazim (as Bavistin)	(5)				*Ascomycetes*, *basidiomycetes*	NOEC (conidia germination): 1 mg/L

(Continued)

TABLE 1 | Continued

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal species	Tested fungal taxa	Lowest toxicity value
Bundschuh et al., 2011	Tebuconazole (as FOLICUR®)	(6)	Food choice, species diversity and total fungal biomass	Black alder leaves conditioned for 3 weeks in a clean near-natural stream cut into leave discs and subsequently exposed to the test concentrations for 12 days: (i) 12 h food choice experiments with *Gammarus fossarum*, (ii) conidia morphology, (iii) ergosterol extraction for biomass determination	***Aquatic hyphomycetes:*** *Alatospora aquatica*, *Lemonniera aquatica*, *Fusarium spp*, *Flagellospora fusarioides*, *Clavariopsis aquatica*, *Heliscus tentaculus*, *Flagellospora curvula*, *Tetracladium marchalianum*, *Anguillospora longissima*, *Tricladium angulatum*, *Varicosporium elodeae*, *Heliscella submerses*, *Clavatospora longibrachiata*, *Heliscella quatic*, *Filosporella spp*, *Lemonniera terrestris*, *Campylospora spp*	*Ascomycetes*, *basidiomycetes*	NOEC: <50 μg/L
Dijksterhuis et al., 2011	Carbendazim	(5)	Growth (biomass)	Pure cultures in liquid medium (96 well plates) or on agar plates; growth determined by visual assessment	***Aquatic hyphomycetes:*** *Trichoderma hamatum, Fusarium sporotrichioides, Helicoon richonis, Helicodendron tubulosum* ***Yeasts:*** *Cryptococcus flavescens* ***Oomycetes:*** *Pythium spp* ***Glomeromycetes:*** *Mucor hiemalis*	*Ascomycetes*, *basidiomycetes*	NOEC: *T. hamatum:* 0.26 mg/L *F. sporotrichioides:* 1 mg/L *H. richonis:* - *H. tubulosum:* - *C. flavescens:* 8.2 mg/L *Pythium spp:* ≥5 mg/L *M. hiemalis:* ≥8.2 mg/L
	Chlorothalonil	(7)				Not specified	NOEC: *T. hamatum:* ≥0.26 mg/L *F. sporotrichioides:* ≥0.26 mg/L *H. richonis:* - *H. tubulosum:* - *C. flavescens:* ≥0.26 mg/L *Pythium spp:* ≥0.2 mg/L *M. hiemalis:* ≥0.26 mg/L
	Fluazinam	(8)				*Ascomycetes*, *basidiomycetes*, *oomycetes*	NOEC: *T. hamatum:* 0.06 mg/L *F. sporotrichioides:* 0.06 mg/L *H. richonis:* - *H. tubulosum:* - *C. flavescens:* 0.06 mg/L *Pythium spp:* 0.1 mg/L *M. hiemalis:* 0.06 mg/L
	Imazalil	(6)				*Ascomycetes*, *basidiomycetes*	NOEC: *T. hamatum:* 0.41 mg/L *F. sporotrichioides:* 3.3 mg/L *H. richonis:* 0.5 mg/L *H. tubulosum:* 0.1 mg/L *C. flavescens:* 26 mg/L *Pythium spp:* 0.1 mg/L *M. hiemalis:* 0.1 mg/L

(Continued)

TABLE 1 | Continued

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal species	Tested fungal taxa	Lowest toxicity value
	Epoxiconazole	(6)				*Ascomycetes*, *basidiomycetes*, *glomeromycetes ascomycetes*, *basidiomycetes*	NOEC: *T. hamatum:* <0.001 mg/L *F. sporotrichioides:* <0.001 mg/L *H. richonis:* 1.2 mg/L *H. tubulosum:* 0.2 mg/L *C. flavescens:* <0.001 mg/L *Pythium spp:* >10 mg/L *M. hiemalis:* >10 mg/L
	Tebuconazole	(6)				*Ascomycetes*, *basidiomycetes*	NOEC: *T. hamatum:* 0.008 mg/L *F. sporotrichioides:* 0.13 mg/L *H. richonis:* 0.5 mg/L *H. tubulosum:* 0.5 mg/L *C. flavescens:* 0.008 mg/L *Pythium spp:* >10 mg/L *M. hiemalis:* >10 mg/L
	Azoxystrobin	(9)				*Ascomycetes*, *basidiomycetes*, *oomycetes*	NOEC: *T. hamatum:* 0.46 mg/L *F. sporotrichioides:* 0.029 mg/L *H. richonis:* >5 mg/L *H. tubulosum:* >5 mg/L *C. flavescens:* 0.46 mg/L *Pythium spp:* 0.002 mg/L *M. hiemalis:* 0.23 mg/L
Lin et al., 2012	Metiram (as formulation Polyram®)	(3)	Growth (biomass), species diversity of two dominant species, leaf decomposition	Litter bags with alder leaves (4 weeks conditioned in experimental ditches prior to the experiment)	***Aquatic hyphomycetes:*** *Anguillospora longissima, Tetracladium setigerum*	*Ascomycetes*, *oomycetes*	NOEC Total fungal biomass: ≥324 μg a.i. /L
Artigas et al., 2012	Tebuconazole	(6)	Growth (biomass), community structure, leaf decomposition	*Alnus glutinosa* und *Populus nigra* leaves exposed in litter bags in a control stream, discs were cut and incubated in the lab for 48h to stimulate mycelia growth and sporulation (to serve as inoculum); exposure of fresh *Alnus* and *Populus* leaves in litterbags in glass indoor channels;	Not specified	*Ascomycetes*, *basidiomycetes*	NOEC: < 20 μg/L

(Continued)

TABLE 1 | Continued

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal species	Tested fungal taxa	Lowest toxicity value
			Inoculation: (i) biomass measured as ergosterol content, (ii) community composition determined with molecular biological methods, (iii) measurement of enzyme kinetics				
Dimitrov et al., 2014	Tebuconazole (as formulation Folicur®)	(6)	Structure, leaf decomposition, conidia production, food chain effects (*Gammarus pulex* feeding rate)	*Alnus glutinosa* leaves exposed in fine and coarse mesh litter bags in experimental ditches with standing water; communities were established 25 days before fungicide application. (i) Leaf litter decomposition measured as loss in dry mass, (ii) shredder feeding rate with *Gammarus pulex* and *Asellus aquaticus*, (iii) fungal and bacterial community composition on leaf litter and sediment (PCR analysis)	Natural fungal communities dominated by *Chytridiomycota* and *Ascomycota*. Dominant genera: ***Aquatic hyphomycetes:*** *Anguillospora, Pestalotiopsis (*both *Ascomycota)* ***Chytridiomycota:*** *Nowakowskiella*, *Cladochytrium*, With low abundance: *Tetracladium, Nectria (*both *Ascomycota)* Conidia production mainly by *Anguillospora longissima* and *Tetracladium setigerum (*both *Ascomycota)*	*Ascomycetes, basidiomycetes*	NOEC: <238 μg/L
Flores et al., 2014	Imazalil	(6)	Sporulation and community composition, number of fungal species	Source of the natural hyphomycete community: *Alnus glutinosa* leaves exposed non-polluted stream Conditioning of leaves in the presence of fungicides: Over 1 week in stream water	*Alatospora acuminate, Alatospora pulchella, Anguillospora rosea, Articulospora proliferate, Articulospora tetracladia, Clavariopsis quatic, Clavatospora longibrachiata, Culicidospora quatic, Flagellospora curvula, Flagellospora sp,. Heliscella stellate, Heliscus lugdunensis, Lunulospora curvula, Stenocladiella neglecta, Tetrachaetum elegans, Tricladium angulatum, Tricladium chaetocladium, Tricladium splendens, Tricladium marchalianum, Tricladium monosporus, Tricladium setigerum*	*Ascomycetes*	NOEC: 0.1 μg/L (*Lunulospora curvula* sporulation)

(Continued)

TABLE 1 | Continued

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal species	Tested fungal taxa	Lowest toxicity value
Zubrod et al., 2015b	Azoxystrobin (as Ortiva)	(9)	Functional endpoints: Fungal biomass and bacterial density; Microbial decomposition of leave material conditioned in the presence of the respective fungicide or fungicide mixture; Feeding of *Gammarus fossarum* on conditioned leaves Structural endpoints: Fungal species per sample, fungal community composition, fungal spore production	Source of the natural hyphomycete community: *Alnus glutinosa* leaves exposed in fine mesh bags in a creek upstream of any agricultural activity settlement or wastewater inlet Mixing with double amount of *Alnus glutinosa* leaves to establish leaves for inoculation Conditioning of leaves in the presence of fungicides: 16 mm leaf discs were incubated over 12 days at 16°C with natural community from inoculum leaves Functional endpoints: Discs were rinsed with fungicide free medium before the tests Structural endpoints: Induction of sporulation in deionised water at 20°C	**Aquatic hyphomycetes** present in experiments with all fungicides: *Alatospora acuminate, Clavariopsis quatic, Clavatospora longibrachiata, Flagellospora curvula, Heliscella quatic, Tetracladium marchalianum, Tricladium angulatum* Aquatic hyphomycetes present in at least one experiment: *Alatospora constricta, Anguillospora crassa,,Anguillospora longissima, Articulospora tetracladia, Geniculospora aquatica, Heliscus lugdunensis, Lemonniera aquatica, Lemonniera terrestris, Lunulospora curvula, Microstella pluvioriens, Mycocentrospora clavata, Naiadella fluitans, Pseudoanguillospora stricta, Sigmoidea aurantiaca, Tetracladium setigerum, Tricladium gracile, Tricldium patulum, Tricldadium terrestre, Tripospermum myrti, Triscelophorus monosporus*	*Ascomycetes*, *basidiomycetes*, *oomycetes*	NOEC: Microbial decomposition: 20 μg/L Fungal biomass: 100 μg/L
	Carbendazim (as Derosal)	(5)				*Ascomycetes*, *basidiomycetes*	NOEC: Microbial decomposition: 35 μg/L Fungal biomass: ≥1715 μg/L
	Cyprodinil (as Chorus)	(10)				*Ascomycetes*	NOEC: Microbial decomposition: 40 μg/L Fungal biomass: 8 μg/L
	Quinoxyfen (as Fortress 250)	(11)				*Ascomycetes*	NOEC: Microbial decomposition: ≥2560 μg/L Fungal biomass: <5 μg/L

(Continued)

TABLE 1 | Continued

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal species	Tested fungal taxa	Lowest toxicity value
	Tebuconazole (as Folicur)	(6)				*Ascomycetes*, *basidiomycetes*	NOEC: Microbial decomposition: ≥500 μg/L Fungal biomass: 1 μg/L
	As well as a mixture of all fungicide formulations						NOEC: Microbial decomposition: 60 μg/L Fungal biomass: 60 μg/L
Zubrod et al., 2015c	Mixture of azoxystrobin, carbendazim, cyprodinil, quinoxyfen, tebuconazole	(5, 6, 9, 10, 11)	Food palatability and feces production (*Gammarus fossarum*), sporulating fungal species per sample	Natural communities on *Alnus glutinosa* leaves, conditioning in the presence or absence of the fungicide mixture, exposure of *G. fossarum* to the fungicide mixture food and or water	*Inter alia Heliscus lugdunensis and Tetracladium marchalianum*	*Ascomycetes*	NOEC: <62.5 μg/L
Abelho et al., 2016	Pyrimethanil	(10)	Growth (biomass, ergosterol concentration)	Litter bags with *Alnus glutinosa* and biofilm pellets	Not specified	*Ascomycetes*	NOEC: <0.73 mg/L (fungal biomass)
Donnadieu et al., 2016	Tebuconazole	(6)	Fungal and bacterial biomass, spores	Indoor stream *Fagus sylvatica leaves* and natural sand	*Lunulospora curvula, Lemonniera aquatica, Clavariopsis aquatica, Diplocladiella scalaroides, Margaritispora aquatica*	*Ascomycetes*	NOEC: <12 μg/L (fungal biomass)
Pesce et al., 2016	Tebuconazole	(6)	Growth (biomass), species diversity	Indoor channels; litter bags with *Alnus glutinosa* were colonized in a pristine area of the Ardières River (France)	e.g., *Anguillospora longissima, Clavariopsis aquatica, Tetracladium marchalianum*	*Ascomycetes*	NOEC: ≥20 μg/L

Functional effects addressed in the same study are also reported.

Modes of action (Tomlin, 2009): *(1) Not specified; (2) Reacts with, and inactivates, the sulfhydryl groups of amino acids and enzymes of fungal cells, resulting in disruption of lipid metabolism, respiration, production of ATP; (3) Non-specific thiol reactant, inhibiting respiration and germination of spores; (4) Ergosterol biosynthesis inhibitor, by inhibition of sterol reduction and isomeration; (5) Beta-tubulin synthesis inhibitor; inhibition development of the germ tubes, the formation of appressoria and the growth of mycelia; (6) Ergosterol biosynthesis inhibitor; (7) Conjugation with, and depletion of thiols (particularly glutathione) from germinating fungal cells, leading to disruption of glycosis and energy production; (8) Uncouples mitochondrial oxidative phosphorylation, inhibiting spore germination, hyphal penetration, growth and sporulation; (9) Inhibition of mitochondrial respiration by blocking electron transfer between cytochrome b and cytochrome* c_1*, at the ubiquinol oxidizing site; Inhibition of spore germination, mycelial growth and antisporulant activity; (10) "proposed inhibitor of the biosynthesis of methionine and the secretion of hydrolytic enzymes"; (11) "growth signal inhibitor."*

TABLE 2 | Summary of literature on freshwater fungi bioassays focussing on functional effects of fungicides.

References	Tested fungicides	Mode of action	Endpoint	Test setup	Tested fungal taxa	Toxicity value
Cuppen et al., 2000	Carbendazim (as Formulation Derosal®)	(5)	Decomposition (Dry weight)	Litter bags with *Populus* leaves and *Elodea* shoots	*Ascomycetes*, *basidiomycetes*	NOEC (dry weight): 100 μg/L
Heimbach et al., 2002	Tolylfluanid (as formulation Euparen M WG50)	(3)	Decomposition rate	Litter bags with *Populus* leaves	*Ascomycetes*, *oomycetes*	NOEC: ≥214 μg a.i./L
Roessink et al., 2006	Triphenyltin	unspecific	Decomposition (Dry weight)	Litter bags with *Populus* leaves		NOEC: ≥100 μg/L
van Wijngaarden et al., 2010	Fluazinam	(8)	Decomposition (Dry weight)	Litter bags with *Populus* leaves	*Ascomycetes*, *basidiomycetes*, *oomycetes*	NOEC: 50 μg/L
Gustafsson et al., 2010	Azoxystrobin	(9)	Decomposition (Dry weight)	Litter bags with *Ranunculus baudotii* stems and leaves	*Aascomycetes*, *basidiomycetes*, *oomycetes*	NOEC: ≥60 μg/L
Willming and Maul, 2016	Pyraclostrobin	(9)	Leaf shredding by *Hyalella azteca*	*H. azteca* feeding on disks of *Acer saccharum* leaves either exposed via the water or via pyraclostrobin conditioned leaves	*Not specified*	NOEC (water exposure): 20 μg/L NOEC (leaf exposure): ≥80 μg/L

*Modes of action (**Tomlin, 2009**): see footnotes of **Table 1**.*

Structural as well as functional endpoints were quantified by Lin et al. (2012) who studied the effects of metiram in outdoor freshwater microcosms on invertebrates, primary producers and microbes. They found no evidence for adverse effects on the biomass and leaf decomposition of aquatic fungi at tested concentrations (0–324 μg/l metiram). No effect on species abundance was observed, however, species identification was limited to only two dominant hyphomycetes species. In the same year, Artigas et al. (2012) conducted a study on the effects of the fungicide tebuconazole on the biomass, community structure, and extracellular enzymatic activities of the microbial community on leaves (*Populus nigra, Alnus glutinosa*) in indoor stream channels. Tebuconazole applied at 33.1 μg/l reduced leaf litter breakdown rates and biomass development, and modified the fungal community. Moreover, shifts in extracellular enzyme activity were observed, resulting in lower cellulose and hemicellulose decomposition in leaves.

Bundschuh et al. (2011) were the first to study structural (i.e., community composition) as well as ecological (i.e., grazing) endpoints and hence added ecological complexity to their test systems. They investigated the effects of the fungicide tebuconazole (applied as FOLICUR®) on the conditioning process of leaf material by means of food-choice experiments with *Gammarus fossarum*. Results showed that gammarids preferred leaves conditioned without fungicide over those conditioned in the presence of the fungicide. In addition, fungal biomass (measured as ergosterol concentration) decreased with increasing fungicide concentration and fungal biodiversity was lower in the presence of 50 μg/l and 500 μg/l tebuconazole. The study of Bundschuh et al. (2011) demonstrates the importance of aquatic fungi as food source for invertebrates in the food web of freshwater ecosystems. Also, Zubrod et al. (2015b) observed a significant influence on the feeding rate of *G. fossarum* when fed leaves preconditioned in the presence of tebuconazole (again applied as FOLICUR®) at a concentration of 500 μg tebuconazole/l. This correlates with shifts in fungal community structure which were observed at 50 μg tebuconazole/l, but were significant only at the next higher test concentration (500 μg/l). Fungal biomass was affected at 5 μg/l whereas the functional endpoint microbial decomposition of leaf material was affected at a concentration of 1 μg/l. The authors also tested formulations of azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and a mixture of all five fungicide formulations (see also Zubrod et al., 2015c) for the same functional and structural endpoints. For four out of five fungicide formulations structural endpoints were more sensitive than functional ones. Similar observations were made by Flores et al. (2014) for the azole fungicide Imazalil and *Echinogammarus berilloni*, albeit at higher concentrations. The number of fungal species was significantly reduced at 100 μg/l, but there were no significant effects on total sporulation. And also a third amphipod species, *Gammarus pulex,* showed a significantly reduced feeding rate when fed azole fungicide exposed leaves (again tebuconazole) (Dimitrov et al., 2014). Willming and Maul (2016) observed a reduction in leaf shredding of the amphipod, *Hyallela azteca,* at 15 μg pyraclostrobin/l. No effect on feeding was observed, however, when only the leaves were exposed to pyraclostrobin, a strobilurin fungicide.

Talk et al. (2016) studied the effects of a mixture of plant protection products, applied in apple orchards. The authors applied the organic fungicides dithianon, dodine, captan, and trifloxystrobin together with copper oxychloride, several insecticides, and herbicides at low concentrations (at or below

their regulatory acceptable concentration) in pond mesocosms and studied the fungal community composition by molecular fingerprinting. However, no significant effects were observed due to the pesticide application. Because of the simultaneous presence of insecticides and herbicides this study is not listed in **Table 1**.

Several studies were conducted on leaf litter decomposition in experimental ponds (**Table 2**). The fungal community structure was not studied in these experiments. In five of 8 studies no treatment related effects on leaf litter decomposition were observed (Heimbach et al., 2002; Roessink et al., 2006; Gustafsson et al., 2010; Lin et al., 2012): azoxystrobin up to 60 μg/l, metiram up to 324 μg/l, triphenyltin acetate up to 100 μg/l. Transient effects were observed in the remaining studies. Cuppen et al. (2000) detected effects on residual dry weights of *Populus* leaves after 4 weeks but not after 2 or 8 weeks, indicating a delayed decay at 330 μg/l and 1,000 μg/l carbendazim. In a mixture toxicity study with the fungicide fluazinam, the insecticide lambda-cyhalothrin, and the herbicides asulam and metamitron (van Wijngaarden et al., 2004), delayed leaf decomposition was observed at day 50 for the three highest tested mixture concentrations, but not after 22 or 92 days of exposure. Because of the presence of insecticides this study is not listed in **Table 2**. For fluazinam applied as a single substance, transient effects for concentrations ≥ 50 μg/l was observed (van Wijngaarden et al., 2010). Pesce et al. (2016) studied the combined effects from fungicide exposure and drought. While a tebuconazole concentration of 20 μg/l did not have a significant effect on leaf litter decomposition when applied alone, it increased the drought effect.

DISCUSSION

Our review clearly demonstrates that fungi are an integral and important part of freshwater ecosystems. Fungicides, which are designed to disrupt fungal cells and their reproduction, have been shown to contaminate surface water bodies in both agricultural and urban areas, and concentrations are high enough to cause concern with regard to negative effects on fungal species and their ecological functions.

Information on the effects of fungicides and fungicide mixtures on fungi is still scarce, primarily because no standardized toxicity tests with fungi species exist. However, non-standard tests have been used in research, including tests with functional (e.g., leaf litter breakdown) and structural (e.g., fungal community composition) endpoints. Results of available studies show that functional test endpoints were generally less sensitive to fungicides than structural endpoints. Mesocosm studies in which leaf litter breakdown was used as an endpoint never showed a long-lasting significant effect in response to fungicide exposure. This is in line with the findings of Cafaro (2005) and Cus et al. (2013), who observed that a decrease in species number did not result in decreased litter breakdown rates. They found, however, that the variability of the litter breakdown rates increased with decreasing species richness. This confirms conclusions made by Bundschuh et al. (2011), who showed that amphipods prefer certain hyphomycete species as food over other fungi, namely that assessing structure is important when aiming at the protection of function.

For several fungicidal modes of action information on fungal toxicity is completely missing so far, e.g., inhibition of nucleic acid synthesis (e.g., metalaxyl-M), inhibition of lipid synthesis (e.g., propamocarb) or cell wall biosynthesis (e.g., dimethomorph). On the other hand, inhibition of sterol biosynthesis (e.g., tebuconazole), inhibition of mitochondrial respiration (e.g., azoxystrobin) and inhibition of beta tubulin synthesis (e.g., carbendazim) are comparatively well studied (**Tables 1**, **2**). It would be desirable to expand the spectrum of test substances and modes of action in future studies.

Currently, the rather qualitative nature of many of the published fungal toxicity studies as well as the limited substance spectrum precludes the performance of a risk assessment, i.e., the comparison of environmental concentrations to effect concentrations. Similarly, there is not enough data to compare sensitivities of aquatic fungi and standard test organisms to fungicides. So far, few assays were able to establish concentration-response curves. Studies aiming at detecting significant differences relative to a control often resulted in unbound (i.e., "<" or "≥") NOECs. In other cases, NOECs were of limited regulatory value because a spacing factor of 10 was used between test concentrations. The fact that formulation additives, which may increase the aquatic toxicity of pesticides (e.g., Coors and Frische, 2011), are usually neither disclosed by the producer nor included in environmental monitoring campaigns, further complicates risk assessments for aquatic fungi. Nevertheless, when the lowest NOEC values (**Tables 1**, **2**) are compared to the highest concentrations detected by Petersen et al. (2013), and Moschet et al. (2014), the resulting toxicity exposure ratios (TER) are 12 and 25 for the triazole, tebuconazole, and the strobilurin, azoxystrobin, respectively. Using the highly resolved exposure data from the study of Spycher et al. (2018) the lowest TER for azoxystrobin is 0.67, indicating a NOEC exceedance. This confirms the conclusions of previous studies, i.e., that effects of fungicides on aquatic fungi may be of regulatory concern (e.g., Bundschuh et al., 2011; Dijksterhuis et al., 2011; Dimitrov et al., 2014; Donnadieu et al., 2016; Feckler et al., 2016).

The need for new methods has been identified in the aquatic risk assessment guidance document for authorization of plant protection products (EFSA, 2013). Based on the protection goals, tests with functional endpoints were encouraged as a possible way forward. Currently, either leaf discs or whole leaves with naturally occurring or previously inoculated fungal communities are exposed in the lab or as so-called "litter bags" in mesocosm studies. Besides the questions regarding their sensitivity, a principal issue with such bioassays is that they are conducted under conditions that hardly possess similarities to those occurring in freshwater ecosystems. For example, studies using litter bags are often performed in ponds rather than streams, where leaf litter breakdown is ecologically more important. For this reason, guidelines should be developed which consider the

ecological relevance of the test system with regard to endpoints and application scenarios. For instance, aquatic *hyphomycetes,* typical species found on submerged leaf litter, should be tested in mesocosms under flow-through conditions, where oxygen concentrations are representative of their preferred habitat (e.g., Donnadieu et al., 2016; Pesce et al., 2016). For stagnant waters, aquatic fungi colonizing standing-dead emergent plants such as cane (predominantly aero-aquatic fungi and yeasts), would be ecologically relevant test organisms. To date, no such bioassay exists, but some methods are described in the literature on aquatic fungi ecology.

While the degradation of dead plant material represents a key function in food webs of freshwater ecosystems, it is known that aquatic fungi fulfill additional important functions which may be at risk due to fungicide exposure, in particular mutualism (Wilson et al., 2014), the control of phytoplankton population dynamics and the degradation of non-plant dead material. Other interactions such as the relationship between enzyme producing microbes and those that profit from these enzymes and may even outgrow the enzyme producing microbes, so called "cheaters" (Allison, 2005), may also be affected. However, this would require relatively complex testing conditions, and no suitable bioassays currently exist to test toxic effects on these functions. A bioassay using chytridiomycetes, a group known to be crucial for the control of phytoplankton populations, and important for nutrient transfer across different trophic levels, would need to simulate the pelagic zone of a lake with a simple food web. Promising methods as a basis for bioassay development for chytridiomycetes can be found in the literature on aquatic fungi ecology. For example, Kagami et al. (2007) tested the control of algal growth by chytridiomycetes, which have already been used to build a population dynamics model for the control of algal blooms by chytridiomycetes.

According to their protection goals, the WFD (EU 2000) and the biocidal products regulation (European Chemicals Agency, 2015) not only aim at protecting functions, but also the structure (biodiversity and abundance) of organisms in freshwater ecosystems (c.f. SI). From an ecological point of view protecting structural diversity is likely to concomitantly protect ecosystem function. Less structurally diverse communities tend to be more vulnerable to chemical and non-chemical stressors (e.g., Vinebrooke et al., 2004; Morin et al., 2015; Pesce et al., 2016). However, identifying fungal diversity means being able to identify species and reliable identification is often difficult (Krauss et al., 2011). Surveys using DNA barcoding and next generation sequencing techniques are promising approaches to depict fungal species structure in freshwater ecosystems and mesocosms.

CONCLUSIONS AND OUTLOOK

Freshwater ecosystems comprise complex food webs in which each species plays an essential role as primary producer (e.g., algae) consumer (e.g., *Daphnia*, fish) or decomposer (e.g., bacteria, fungi). Although largely understudied, aquatic fungi fulfill important and unique functions in freshwater ecosystems, especially in the degradation of allochthonous dead plant litter and the resulting energy transfer to higher trophic levels. In addition, recent studies demonstrate their importance in population dynamics of phytoplankton. Other ecological roles of freshwater fungi may yet be discovered.

The biodiversity and abundance of fungal communitieg9s in freshwater ecosystems is not explicitly protected by current EU regulation. Due to their important ecosystem functions, it is obvious that aquatic fungi should be considered when assessing the risk of pesticides—especially fungicides, of which they are the target organisms. There is evidence that triazoles, in particular, can adversely affect the fungal community of freshwater ecosystems (Bundschuh et al., 2011; Artigas et al., 2012) at environmentally relevant concentrations (Donnadieu et al., 2016). We therefore recommend to extend fungicide risk assessment for aquatic organisms to the trophic level of decomposers using selected fungal species as test organisms. Sufficiently developed methods are available for leaf litter decomposing hyphomycetes. They are of high relevance and should be used in current fungicide risk assessments. In parallel, new fungal bioassays should be developed to account for the structural and functional diversity of aquatic fungi, e.g., interactions of chytridiomycetes with algae and their effect on algal population growth, and fungicide effects on trichomycetes living in the guts of aquatic arthropods.

AUTHOR CONTRIBUTIONS

LI and MJ conducted the review and would therefore like to share first authorship. IW supervised the work and was involved in the design und preparation of the review.

FUNDING

The study was solely funded by the Swiss Center for Applied Ecotoxicology Eawag-EPFL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2018.00105/full#supplementary-material

REFERENCES

Abelho, M., Martins, T. F., Shinn, C., Moreira-Santos M., and Ribeiro R. (2016). Effects of the fungicide pyrimethanil on biofilm and organic matter processing in outdoor lentic mesocosms. *Ecotoxicology* 25, 121–131. doi: 10.1007/s10646-015-1574-x

Adl, S. M., Simpson, A. G., Farmer, M. A., Anderson, R. A., Anderson, O. R., Barta, J. R., et al. (2005). The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukary Microbiol.* 52, 399–451. doi: 10.1111/j.1550-7408.2005.00053.x

Ahearn, D. G., Roth, F. J., and Meyers, S. P. (1968). Ecology and characterization of yeasts from aquatic regions of

South Florida. *Mar. Biol.* 1, 291–308. doi: 10.1007/BF00360780

Allison, S. D. (2005). Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments. *Ecol. Lett.* 8, 626–635. doi: 10.1111/j.1461-0248.2005.00756.x

Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., et al. (2012). Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquat. Toxicol.* 122–123, 197–205. doi: 10.1016/j.aquatox.2012.06.011

Augustin, T., Schlosser, D., Baumbach, R., Schmidt, J., Grancharov, K., Krauss, G., et al. (2006). Biotransformation of 1-naphtol by a strictly aquatic fungus. *Curr. Microbiol.* 52, 216–220. doi: 10.1007/s00284-005-0239-z

Azevedo, M. M., Carvalho, A., Pascoal, C., Rodrigues, F., and Cássio, F. (2007). Responses of antioxidant defenses to Cu and Zn stress in two aquatic fungi. *Sci. Total Environ.* 377, 233–243. doi: 10.1016/j.scitotenv.2007.02.027

Bärlocher, F., Guenzel, K., Sridhar, K. R., and Duffy, S. J. (2011). Effects of 4-n-nonylphenol on aquatic hyphomycetes. *Sci. Total Environ.* 409, 1651–1657. doi: 10.1016/j.scitotenv.2011.01.043

Bärlocher, F., and Kendrick, B. (1974). Dynamics of the fungal populations on leaves in a stream. *J. Ecol.* 62, 761–791. doi: 10.2307/2258954

Bärlocher, F., and Premdas, P. D. (1988). Effects of pentachlorophenol on aquatic hyphomycetes. *Mycologia* 80, 135–137. doi: 10.1080/00275514.1988.12025513

Benny, G. L., and O'Donnell, K. (2000). Amoebidium parasiticum is a protozoan, not a Trichomycete. *Mycologia* 92, 1133–1137. doi: 10.2307/3761480

Bereswil, R., Golla, B., Streloke, M., and Schulz, R. (2012). Entry and toxicity of organic pesticides and copper in vineyard streams: erosion rills jeopardise the efficiency of riparian buffer strips. *Agric. Ecosyst. Environ.* 146 81–92 doi: 10.1016/j.agee.2011.10.010

Bundschuh, M., Zubrod, J. P., Kosol, S., Maltby, L., Stang, C., Duester, L., et al. (2011). Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. *Aquatic Toxicol.* 104, 32–37 doi: 10.1016/j.aquatox.2011.03.010

Cafaro, M. J. (2005). Eccrinales (Trichomycetes) are not fungi, but a clade of protists at the early divergence of animals and fungi. *Mol. Phylogenet. Evol.* 35, 21–34. doi: 10.1016/j.ympev.2004.12.019

Chandrashekar, K. R., and Kaveriappa, K. M. (1989). Effect of pesticides on the growth of aquatic hyphomycetes. *Toxicol. Lett.* 48, 311–315. doi: 10.1016/0378-4274(89)90058-1

Chandrashekar, K. R., and Kaveriappa, K. M. (1994). Effect of pesticides on sporulation and germination of conidia of aquatic hyphomycetes. *J. Environ. Biol.* 15, 315–324.

Coors, A., and Frische, T. (2011). Predicting the aquatic toxicity of commercial pesticide mixtures. *Environ. Sci Europe* 23:22 doi: 10.1186/2190-4715-23-22

Cruzeiro, C., Rocha, E., Pardal, M. Â., and Rocha, M. J. (2015). Uncovering seasonal patterns of 56 pesticides in surface coastal waters of the Ria Formosa lagoon (Portugal), using a GC-MS method. *Int. J. Environ. Anal. Chem.* 95, 1370–1384. doi: 10.1080/03067319.2015.1100724

Cummins, K. W. (1974). Structure and functions of stream ecosystems. *Bio. Sci.* 24, 631–641. doi: 10.2307/1296676

Cuppen, J. G., van den Brink, P. J., Camps, E., Uil, K. F., and Brock, T. C. M. (2000). Impact of the fungicide carbendazim in freshwater microcosms. I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates. *Aquatic Toxicol.* 48, 233–250. doi: 10.1016/S0166-445X(99)00036-3

Cus, F., Bach, B., Barnavon, L., and Pongrac, V. Z. (2013). Analytical determination of Dolenjska region wines quality. *Food Control* 33, 274–280. doi: 10.1016/j.foodcont.2013.03.017

Dijksterhuis, J., van Doorn, T., Samson, R., and Postma, J. (2011). Effects of seven fungicides on non-target aquatic fungi. *Water Air Soil Pollution* 222, 421–425. doi: 10.1007/s11270-011-0836-3

Dimitrov, M. R., Kosol, S., Smidt, H., Buijse, L., Van den Brink, P. J., Van Wijngaarden, R. P., et al. (2014). Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. *Sci. Total Environ.* 490, 1002–1011. doi: 10.1016/j.scitotenv.2014.05.073

Donnadieu, F., Besse-Hoggan, P., Forestier, C., and Artigas, J. (2016). Influence of streambed substratum composition on stream microbial communities exposed to the fungicide tebuconazole. *Freshw. Biol.* 61, 2026–2036. doi: 10.1111/fwb.12679

Duddridge, J. E., and Wainwright, M. (1980). Heavy metal accumulation by aquatic fungi and reduction in viability of *Gammarus pulexi* fed Cd^{2+} contaminated mycelium. *Water Res.* 14, 1605–1611. doi: 10.1016/0043-1354(80)90065-2

EFSA (2013). *Outcome of the Public Consultation on the Draft PPR Panel Guidance Document on Tiered Risk Assessment for Plant Protection Products for Aquatic Organisms in Edge-Of-Field Surface Waters.* Technical report EFSA supporting publication 2013: EN−460.

European Chemicals Agency (2015). *Guidance on Biocidal Products Regulation: Volume IV Environment Part B Risk Assessment (active substances).* Helsinki: European Chemicals Agency.

European Commission (2011). *Common Implementation Strategy for the Water Framework Directive*. Technical Guidance for Deriving Environmantal Quality Standards

Feckler, A., Goedkoop, W., Zubrod, J. P., Schulz, R., and Bundschuh, M. (2016). Exposure pathway-dependent effects of the fungicide epoxiconazole on a decomposer-detritivore system. *Sci. Total Environ.* 571, 992–1000. doi: 10.1016/j.scitotenv.2016.07.088

Fernández, D., Voss, K., Bundschuh, M., Zubrod, J. P., and Schäfer, R. B. (2015). Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. *Sci. Total Environ.* 533, 40–48 doi: 10.1016/j.scitotenv.2015.06.090

Findlay, S., and Arsuffi, T. L. (1989). Microbial growth and detritus transformations during decomposition of leaf litter in a stream. *Freshwater Biol* 21, 261–269. doi: 10.1111/j.1365-2427.1989.tb01364.x

Fisher, S., and Likens, G. (1973). Energy flow in Bear Brook, New Hamphshire: an intergrative approach to stream ecosystem metabolism. *Ecol. Monogr.* 43, 421–439. doi: 10.2307/1942301

Flores, L., Banjac, Z., Farré, M., Larrañaga, A., Mas-Martí, E., Muñoz, I., et al. (2014). Effects of a fungicide (imazalil) and an insecticide (diazinon) on stream fungi and invertebrates associated with litter breakdown. *Sci. Total Environ.* 476–477, 532–541. doi: 10.1016/j.scitotenv.2014.01.059

Gardeström, J., Ermold, M., Goedkoop, W., and McKie, B. G. (2016). Disturbance history influences stressor impacts: effects of a fungicide and nutrients on microbial diversity and litter decomposition. *Freshw. Biol.* 61, 2171–2184. doi: 10.1111/fwb.12698

Gessner, M. O., Chauvet, E., and Dobson, M. (1999). A perspective on leaf litter breakdown in streams. *Oikos* 85, 377–384. doi: 10.2307/3546505

Gessner, M. O., Gulis, V., Kuehn, K. A., Chauvet, E., and Suberkropp, K. (2007). "Fungal Decomposers of Plant Litter in Aquatic Ecosystems," in *The Mycota*, ed K. Esser (Berlin: Springer), 301–324.

Gleason, F. H., Maiko, K., Lefevre, E., and Sime-Ngando, T. (2008). The ecology of chytrids in aquatic ecosystems: roles in food web dynamics. *Fung. Biol. Rev.* 22, 17–25. doi: 10.1016/j.fbr.2008.02.001

Goh, T. K., and Hyde, K. D. (1996). Biodiversity of freshwater fungi. *J. Ind. Microbiol. Biot.* 17, 328–345. doi: 10.1007/BF01574764

Gustafsson, K., Blidberg, E., Elfgren, I. K., Hellström, A., Kylin, H., and Gorokhova, E. (2010). Direct and indirect effects of the fungicide azoxystrobin in outdoor brackish water microcosms. *Ecotoxicology* 19, 431–444. doi: 10.1007/s10646-009-0428-9

Hawksworth, D. L. (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycol. Res.* 95, 641–655. doi: 10.1016/S0953-7562(09)80810-1

Hawksworth, D. L. (2001). The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycol. Res.* 105, 1422–1432. doi: 10.1017/S0953756201004725

Heimbach, F., Brock, T., Arts, G., and Deneer, J. (2002). Effects of multiple applications of Tolylfluanid WG50 on the aquatic community in outdoor microcosm enclosures. Bayer, A. G., unpublished report No.:HBF/Mt 13 cited in the EU Draft Assessment Report (DAR). for Tolylfluanid Volume 3, Annex, B., B.9 Ecotoxicology, 480–482.

Hernández Roa, J. J., Virella, C. R., and Cafaro, M. J. (2009). First survey of arthropod gut fungi and associates from Vieques, Puerto Rico. *Mycologia* 101, 896–903. doi: 10.3852/08-187

Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., et al. (2007). A higher-level phylogenetic classification of the Fungi. *Mycol. Res.* 111, 509–547. doi: 10.1016/j.mycres.2007.03.004

Inoue, S., Igarashi, Y., Yoneda, Y., Kawai, S., Okamura, H., and Nishida, T. (2015). Elimination and detoxification of fungicide miconazole and antidepressant sertraline by manganese peroxidase-dependent lipid peroxidation system. *Int. Biodeteriorat. Biodegradat.* 100, 79–84. doi: 10.1016/j.ibiod.2015.02.017

Jaeckel, P., Krauss, G., Menge, S., Schierhorn, A., Rücknagel, P., and Krauss, G. J. (2005a). Cadmium induces a noval metallothionein and phytochelatin 2 in an aquatic fungus. *Biochem. Bioph. Res. Co.* 333, 150–155. doi: 10.1016/j.bbrc.2005.05.083

Jaeckel, P., Krauss, G.-J., and Krauss, G. (2005b). Cadmium and zinc response of the *Heliscus lugdenensis* and *Verticillium* cf. *alboatrum* isolated from highly polluted water. *Sci. Total Environ.* 346, 274–279. doi: 10.1016/j.scitotenv.2004.12.082

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., et al. (2006). Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* 443, 818–822. doi: 10.1038/nature05110

Jobard, M., Rasconi, S., and Sime-Ngando, T. (2010). Diversity and functions of microsporic fungi: a missing component in pelagic food webs. *Aquat. Sci.* 72, 255–268. doi: 10.1007/s00027-010-0133-z

Kagami, M. (2008). The roles of parasitic chytrids in the aquatic food web. *Jpn. J. Ecol.* 58, 71–80.

Kagami, M., de Bruin, A., Ibelings, B. W., and Van Donk, E. (2007). Parasitic chytrids: their effects on phytoplankton communities and food-web dynamics. *Hydrobiologia* 578, 113–129. doi: 10.1007/s10750-006-0438-z

Kagami, M., Miki, T., and Takimoto, G. (2014). Mycoloop: Chytrids in aquatic food webs. *Front. Microbiol.* 5:166. doi: 10.3389/fmicb.2014.00166

Krauss, G.-J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., and Bärlocher, F. (2011). Fungi in freshwaters: ecology, physiology and biochemical potential. *FEMS Microbiol. Rev.* 35, 620–651. doi: 10.1111/j.1574-6976.2011.00266.x

Kreuger, J., Graaf, S., Patring, J., and Adielsson, S. (2010). Pesticides in surface water in areas with open ground and greenhouse horticultural crops in Sweden 2008. *Ekohydrologi* 117, 1–47.

Lichtwardt, R. W., and Williams, M. C. (1999). Three Harpellales that live in one species of aquatic chironomid larva. *Mycologia* 91, 396–399. doi: 10.2307/3761385

Lin, R., Buijse, L., Dimitrov, M. R., Dohmen, P., Kosol, S., Maltby, L., et al. (2012). Effects of the fungicide metiram in outdoor freshwater microcosms: responses of invertebrates, primary producers and microbes. *Ecotoxicology* 21, 1550–1569. doi: 10.1007/s10646-012-0909-0

Lucas, D., Badia-Fabregat, M., Vicent, T., Caminal, G., Rodríguez-Mozaz, S., Balcázar, J. L., et al. (2016). Fungal treatment for the removal of antibiotics and antibiotic resistance genes in veterinary hospital wastewater. *Chemosphere* 152, 301–308. doi: 10.1016/j.chemosphere.2016.02.113

Lutzoni, F., Kauff, F., Cox, C. J., McLaughlin, D., Celio, G., Dentinger, B., et al. (2004). Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *Am. J. Bot.* 91, 1446–1480. doi: 10.3732/ajb.91.10.1446

Maltby, L., Brock, T. C., and van den Brink, P. J. (2009). Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. *Environ. Sci. Technol.* 43, 7556–7563. doi: 10.1021/es901461c

Martínková, L., Kotik, M., Marková, E., and Homolka, L. (2016). Biodegradation of phenolic compounds by Basidiomycota and its phenol oxidases: a review. *Chemosphere* 149, 373–382. doi: 10.1016/j.chemosphere.2016.01.022

Massaccesi, G., Romero, M. C., Cazau, M. C., and Bucsinszky, A. M. (2002). Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina). *World J. Microb. Biot.* 18, 817–820. doi: 10.1023/A:1021282718440

Miki, T., Takimoto, G., and Kagami, M. (2011). Roles of parasitic fungi in aquatic food webs: a theoretical approach. *Freshwater Biol.* 56, 1173–1183. doi: 10.1111/j.1365-2427.2010.02562.x

Molander, S., Blanck, H., and Söderström, M. (1990). Toxicity assessment by pollution-induced community tolerance (PICT), and identification of metabolites in periphyton communities after exposure to 4,5,6-trichloroguaiacol. *Aquatic Toxicol.* 18, 115–136. doi: 10.1016/0166-445X(90)90022-H

Morin, S., Bonet, B., Corcoll, N., Guasch, H., Bottin, M., and Coste, M. (2015). Cumulative stressors trigger increased vulnerability of diatom communities to additional disturbances. *Microbiol. Aquat. Syst.* 70, 585–595. doi: 10.1007/s00248-015-0602-y

Moschet, C., Wittmer, I., Simovic, J., Junghans, M., Piazzoli, A., Singer, H., et al. (2014). How a complete pesticide screening changes the assessment of surface water quality. *Environ. Sci. Technol.* 48, 5423–5432. doi: 10.1021/es500371t

Mueller, G. M., and Schmit, J. P. (2007). Fungal biodiversity: what do we know? What can we predict? *Biodivers. Conserv.* 16, 1–5. doi: 10.1007/s10531-006-9117-7

Müller-Navarra, D. C., Brett, M. T., Liston, A. M., and Goldman, C. R. (2000). A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature* 403, 74–77. doi: 10.1038/47469

Nelson, D. J., and Scott, D. C. (1962). Role of detritus in the productivity of a rock-outcrop community in a Piedmont stream. *Limnol. Oceanogr.* 7, 396–413. doi: 10.4319/lo.1962.7.3.0396

Nowell, L. H., Moran, P. W., Schmidt, T. S., Norman, J. E., Nakagaki, N., Shoda, M. E., et al. (2018). Complex mixtures of dissolved pesticides show potential aquatic toxicity in a synoptic study of Midwestern U.S. streams. *Sci. Total Environ.* 613–614, 1469–1488. doi: 10.1016/j.scitotenv.2017.06.156

Oliveira, B. R., Penetra, A., Cardoso, V. V., Benoliel, M. J., Barreto Crespo, M. T., Samson, R. A., et al. (2015). Biodegradation of pesticides using fungi species found in the aquatic environment. *Environ. Sci. Pollut. Res.* 22, 11781–11791. doi: 10.1007/s11356-015-4472-0

Oliveira, M. V., Vidal, B. T., Melo, C. M., de Miranda Rde, C., Soares, C. M., Coutinho, J. A., et al. (2016). (Eco)toxicity and biodegradability of protic ionic liquids. *Chemosphere* 147, 460–466. doi: 10.1016/j.chemosphere.2015.11.016

Omoike, A., Wacker, T., and Navidonski, M. (2013). Biodegradaion of bisphenol A by *Heliscus lugdunensis*, a naturally occurring hyphomycete in freshwater environments. *Chemosphere* 91, 1643–1647. doi: 10.1016/j.chemosphere.2012.12.045

Pascoal, C., Cássio, F., and Marvanov,á, L. (2005). Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Archiv für Hydrobiologie* 162, 481–496. doi: 10.1127/0003-9136/2005/0162-0481

Pesce, S., Zoghlami, O., Margoum, C., Artigas, J., Chaumot, A., and Foulquier, A. (2016). Combined effects of drought and the fungicide tebuconazole on aquatic leaf litter decomposition. *Aquat. Toxicol.* 173, 120–131. doi: 10.1016/j.aquatox.2016.01.012

Petersen, K., Stenrod, M., and Tollefsen, K. E. (2013). *Initial Environmental Risk Assessment of Combined Effects of Palnt Protection Products in Six Different Areas in Norway*. Oslo: NIVA - Norwegian Institute for Water Research.

Rabiet, M., Margoum, C., Gouy, V., Carluer, N., and Coquery, M. (2010). Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment - Effect of sampling frequency. *Environ. Pollut.* 158, 737–748 doi: 10.1016/j.envpol.2009.10.014

Rasconi, S., Grami, B., Niquil, N., Jobard, M., and Sime-Ngando, T. (2014). Parasitic chytrids sustain zooplankton growth during inedible algal bloom. *Front. Microbiol.* 5:229. doi: 10.3389/fmicb.2014.00229

Roessink, I., Crum, S. J. H., Bransen, F., van Leeuwen, E., Van Kerkum, F., Koelmans, A. A., et al. (2006). Impact of triphenyltin acetate in microcosms simulating floodplain lakes. I. Influence of sediment quality. *Ecotoxicology* 15, 267–293. doi: 10.1007/s10646-006-0058-4

Rossi, F., Artigas, J., and Mallet, C. (2017). Structural and functional responses of leaf-associated fungal communities to chemical pollution in streams. *Freshw. Biol.* 62, 1207–1219. doi: 10.1111/fwb.12937

Roussel, H., Chauvet, E., and Bonzom, J. M. (2008). Alteration of leaf decomposition in copper-contaminated freshwater mesocosms. *Environ. Toxicol. Chem.* 27, 637–644. doi: 10.1897/07-168.1

Shearer, C. A., Descals, E., Kohlmeyer, B., Kohlmeyer, J., Marvanov,á, L., Padgett, D., et al. (2007). Fungal biodiversity in aquatic habitats. *Biodivers. Conserv.* 16, 49–67. doi: 10.1007/s10531-006-9120-z

Solé, M., Fetzer, I., Wennrich, R., Sridhar, K. R., Harms, H., and Krauss, G. (2008). Aquatic hyphomycete communities as potential bioindicators

for assessing anthropogenic stress. *Sci. Total Environ.* 389, 557–565. doi: 10.1016/j.scitotenv.2007.09.010

Spycher, S., Mangold, S., Doppler, T., Junghans, M., Wittmer, I., Stamm, C., et al. (2018). Pesticide risks in small streams-how to get as close as possible to the stress imposed on aquatic organisms. *Environ. Sci. Technol.* 52, 4526–4535. doi: 10.1021/acs.est.8b00077

Sridhar, K. R., Bärlocher, F., Wennrich, R., Krauss, G.-J., and Krauss, G. (2008). Fungal biomass and diversity in sediments and on leaf litter in heavy metal contaminated waters of Central Germany. *Fund. Appl. Limnol.* 171, 63–74. doi: 10.1127/1863-9135/2008/0171-0063

Strongman, D. B. (2007). Trichomycetes in aquatic insects from Prince Edward Island, Canada. *Can. J. Bot.* 85, 949–963. doi: 10.1139/B07-095

Sungur, S., and Tunur, C. (2012). Investigation of pesticide residues in vegetables and fruits grown in various regions of Hatay, Turkey. *Food Addit. Cont. Part B-Surveill.* 5, 265–267. doi: 10.1080/19393210.2012.704597

Talk, A., Kublik, S., Uksa, M., Engel, M., Berghahn, R., Welzl, G., et al. (2016). Effects of multiple but low pesticide loads on aquatic fungal communities colonizing leaf litter. *J. Environ. Sci*. 46, 116–125. doi: 10.1016/j.jes.2015.11.028

Teal, J. M. (1957). Community metabolism in a temperate cold spring. *Ecol. Monogr.* 27, 283–302. doi: 10.2307/1942187

Tomlin, C. D. S. (2009). *The Pesticide Manual.* Alton: BCPC.

Van Metre, P. C., Alvarez, D. A., Mahler, B. J., Nowell, L., Sandstrom, M., and Moran, P. (2017). Complex mixtures of pesticides in Midwest U.S. streams indicated by POCIS time-integrating samplers. *Environ. Pollut.* 220, 431–440. doi: 10.1016/j.envpol.2016.09.085

van Wijngaarden, R. P., Arts, G. H., Belgers, J. D., Boonstra, H., Roessink, I., Schroer, A. F., et al. (2010). The species sensitivity distribution approach compared to a microcosm study: a case study with the fungicide fluazinam. *Ecotoxicol. Environ. Saf.* 73, 109–122. doi: 10.1016/j.ecoenv.2009.09.019

van Wijngaarden, R. P., Cuppen, J. G., Arts, G. H., Crum, S. J., van den Hoorn, M. W., Van Den Brink, P. J., et al. (2004). Aquatic risk assessment of a realistic exposure to pesticides used in bulb crops: a microcosm study. *Environ. Toxicol. Chem.* 23, 1479–1498. doi: 10.1897/03-80

Vinebrooke, R. D., Cottingham, K. L., Norberg, J., Scheffer, M., Dodson, S. I., Maberly, S. C., et al. (2004). Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. *Oikos* 104, 451–457. doi: 10.1111/j.0030-1299.2004.13255.x

Voigt, K., and Kirk, P. M. (2011). Recent developments in the taxonomic affiliation and phylogenetic positioning of fungi: impact in applied microbiology and environmental biotechnology. *Appl. Microbiol. Biot.* 90, 41–57. doi: 10.1007/s00253-011-3143-4

Willming, M. M., and Maul, J. D. (2016). Direct and indirect toxicity of the fungicide pyraclostrobin to Hyalella azteca and effects on leaf processing under realistic daily temperature regimes. *Environ. Pollut*. 211, 435–442. doi: 10.1016/j.envpol.2015.11.029

Wilson, E. R., Smalling, K. L., Reilly, T. J., Gray, E., Bond, L., Steele, L., et al. (2014). Assessing the potential effects of fungicides on nontarget gut fungi (trichomycetes). and their associated larval black fly hosts. *J. Am. Water Resour. Assoc*. 50, 420–433. doi: 10.1111/jawr.12166

Woese, C. R., and Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5088–5090. doi: 10.1073/pnas.74.11.5088

Woese, C. R., Kandler, O., and Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4576–4579. doi: 10.1073/pnas.87.12.4576

Wong, M. K. M., Goh, T.-K., Hodgkiss, I. J., Hyde, K. D., Ranghoo, V. M., Tsui, C. K. M., et al. (1998). Role of fungi in freshwater ecosystems. *Biodivers. Conservat.* 7, 1187–1206. doi: 10.1023/A:1008883716975

Wurzbacher, C., Rösel, S., Rychła, A., and Grossart, H. P. (2014). Importance of saprotrophic freshwater fungi for pollen degradation. *PLoS ONE* 9:e94643. doi: 10.1371/journal.pone.0094643

Wurzbacher, C. M., Bärlocher, F., and Grossart, H.-P. (2010). Fungi in lake ecosystems. *Aquat. Microb. Ecol.* 59, 125–149. doi: 10.3354/ame01385

Zubrod, J. P., Englert, D., Feckler, A., Koksharova, N., Konschak, M., Bundschuh, R., et al. (2015b). Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? *Environ. Sci. Technol.* 49, 1173–1181. doi: 10.1021/es5050453

Zubrod, J. P., Englert, D., Wolfram, J., Wallace, D., Schnetzer, N., Baudy, P., et al. (2015c). Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda). *Aquat. Toxicol.* 169, 105–112. doi: 10.1016/j.aquatox.2015.10.008

Zubrod, J. P., Feckler, A., Englert, D., Koksharova, N., Rosenfeldt, R. R., Seitz, F., et al. (2015a). Inorganic fungicides as routinely applied in organic and conventional agriculture can increase palatability but reduce microbial decomposition of leaf litter. *J. Appl. Ecol.* 52, 310–322. doi: 10.1111/1365-2664.12393

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Ittner, Junghans and Werner. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership