



Effect of Three Pesticides Used in Salmon Farming on Ammonium Uptake in Central-Southern and Northern Patagonia, Chile

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Chile is the second largest global producer of farmed salmon. The growth of salmon production has not been free of environmental challenges, such as the increasing use of pesticides to control the parasitic load of the sea lice *Caligus rogercresseyi*. The lack of the specificity of pesticides can potentially affect non-target organisms, as well as the structure and functioning of aquatic ecosystems. The aim of this study, was to understand the effect of pesticides on natural microbial communities to the addition of the anti-lice pesticide azamethiphos, deltamethrin and emamectin benzoate, and their potential impact in ammonium uptake rates in the coast off central-southern Chile and Northern Patagonia. The addition of pesticides on natural microbial communities resulted in a rapid response in ammonium uptake, which was significant for the single use of pesticide, azamethiphos and emamectin benzoate, as well as the combination, azamethiphos, deltamethrin and emamectin benzoate. In northern Patagonia, azamethiphos addition produced a 53% decrease in photoautotrophic uptake. However, an increase, although variable, was observed in chemoautotrophic uptake. Emamectin benzoate produced a 36 to 77% decrease in chemo and photoautotrophic ammonium uptake, respectively. The combined use of pesticides, also produced up to 42% decrease in both photo and chemoautotrophic assimilation. We conclude that the use of pesticides in salmon farming produces diverse responses at the microbial level, stimulating and/or inhibiting microbial communities with subsequent impact on nitrogen budgets. Further studies are necessary to understand the impact of pesticides in the ecology of central-southern and northern Patagonia, Chile.

Keywords: ammonium uptake, salmon farming, azamethiphos, deltamethrin, emamectin benzoate, Chile

INTRODUCTION

Chile is the second largest global producer of farmed salmon, with an annual production of over 700 thousand tons (Avenidaño-Herrera, 2018). The growth of Chilean salmon production has not been free of sanitary and environmental difficulties, such as benthic accumulation of organic matter, eutrophication, massive escapes of farmed salmon, input of chemicals such as antibiotic and

pesticides into the ecosystem, fish diseases, and social issues (Burridge et al., 2010; Hargrave, 2010; Baskett et al., 2013; Dresdner et al., 2019; Quiñones et al., 2019).

The intensive use of pesticides to control sea lice infections of *Caligus rogercresseyi* has become a key environmental concern for Chilean aquaculture (Millanao et al., 2011; Núñez-Acuña et al., 2015; Avendaño-Herrera, 2018). Infestations result in severe skin erosion and epidermal hemorrhage, leading to chronic stress, reduced growth (González and Carvajal, 2003; Rozas and Asencio, 2007; González et al., 2015) and increasing the vulnerability of fish to other diseases (Bravo, 2003; Johnson et al., 2004; Dresdner et al., 2019).

Since the earliest reported infections, sea lice in Chile have been controlled using chemicals originally used in agriculture (Reyes and Bravo, 1983). Feed additives such as emamectin benzoate have been used since the 1990s (Bravo, 2003; Bravo et al., 2008) and later complemented by pyrethroids such as deltamethrin in 2007 due to the appearance of resistance of *C. rogercresseyi* to emamectin benzoate. In 2009, the Chilean authorities allowed the use of another pyrethroid, cypermethrin. Diflubenzuron was used between 2008 and 2012 and finally the organophosphate azamethiphos was introduced in Chile in 2013 (Helgesen et al., 2014).

Currently, the most commonly used products in Chile are the organophosphate azamethiphos and the pyrethroid deltamethrin. They are applied *in situ* by bath solution and are subsequently discharged into the surrounding environment in large volumes (Nash, 2003; Burridge et al., 2010; Gebauer et al., 2017). Helgesen et al. (2014) reported that 677 kg of active compound cypermethrin and 197 kg of deltamethrin were discarded in 2012.

The anti-lice treatments lack specificity and therefore may affect indigenous organisms with the potential to affect sensitive non-target species (Johnson et al., 2004). However, until now the studies available describing the impacts of pesticides in non-target organism are scarce and are mainly focus in invertebrates (Burridge et al., 2004; Knapp et al., 2005; Gebauer et al., 2017). Studies focusing on microorganism are even more scarce, and recently was reported that the use of pesticides in marine waters can produce changes in microbial photo and chemoautotrophic carbon uptake (Rain-Franco et al., 2018).

Microorganism are important habitants of aquatic ecosystems, playing a pivotal role in primary productivity and nutrient recycling. In the traditional view of the marine nitrogen (N) cycle, phytoplankton use dissolved inorganic N as nitrate (NO_3^-) or ammonium (NH_4^+) to create biomass while heterotrophic bacterioplankton remineralize dissolved organic N into the inorganic forms hence supporting primary production. By being in contact with pesticides, microbial community structure and metabolic fluxes can be modified, an effect that varies according to their chemical form and characteristics while interacting with the nutrient pool of the water column (Rain-Franco et al., 2018). This can result in an alteration of biogeochemical fluxes and local primary production.

To understand the impact of pesticides on non-target organisms and biogeochemistry, we investigated the potential effect of three pesticides used against *C. rogercresseyi*

(azamethiphos, deltamethrin, and emamectin benzoate) on photo and chemoautotrophic ammonium uptake in two key regions of the Chilean coast that hold high levels of biological productivity but contrasting levels of influence from salmon farming.

MATERIALS AND METHODS

Study Area and Sampling Strategy

The study was conducted in two sites in central-southern and norther Patagonia, Chile: Llico Bay and Caucahue Channel, respectively. Llico Bay is a close bay located south of Gulf of Arauco in central Chile (37.1°S 73.5°W ; **Figure 1A**) in an area untouched by salmon farming while, Caucahue Channel was located in the inland sea of Chiloe island, Northern Patagonia (42.1°S 73.4°W ; **Figure 1B**) and is intensely influenced by salmon farming. Llico Bay was visited 4 times on board of R/V Kay Kay II (Universidad de Concepción) between July 2015 and April 2016 (**Table 1**). Caucahue Channel was visited twice on board the L/M “Don José” in June 2014 and January 2015 (**Table 1**).

During the sampling, we first assessed physical parameters such as temperature and salinity (CTD Minus; AML Oceanographic, Canada) at the three stations in Llico (**Figure 1** and **Table 1**). During Caucahue Channel sampling, we used a CTDO data sensor (SAIV A/S, Norway; **Figure 1** and **Table 1**). Chemical and biological samples were collected with Niskin bottles at all sampling stations (**Table 1**). Ammonium samples were taken in duplicate using 50 mL glass bottles (Duran Schott) and analyzed by the fluorometric method described by Holmes et al. (1999), using a Turner Designs fluorometer. Samples for nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) were stored in high-density polyethylene (HDPE) plastic bottles (11 mL in duplicate) at -20°C until analyses by standard colorimetric techniques (Aminot and Kérouel, 2007). Bacterioplankton abundance was analyzed by flow cytometry (Marie et al., 1997) at Laboratory for oceanographic Processes and Climate (PROFC; Universidad de Concepción, Chile) with a FACSCalibur flow cytometer (Becton Dickinson). Total chlorophyll-a (Chl-a) were estimated using a Turner design fluorometer according (Holm-Hansen et al., 1965).

Isotopic Analysis and *in situ* Ammonium Uptake Experiments

Rates of ammonium uptake (ρNH_4^+) were determined using the $^{13}\text{C}/^{15}\text{N}$ stable isotope technique (Fernández et al., 2009; Fernandez and Farías, 2012). Results of Carbon-13 tracer were published previously by Rain-Franco et al. (2018), which describes carbon fluxes for the same experiments. For ammonium uptake experiments, seawater samples taken from depths described in **Table 1** were incubated in 600 mL polycarbonate bottles using $^{15}\text{NH}_4\text{Cl}$ (ICON isotopes IN 5037; 99% at $0.5 \mu\text{mol mL}^{-1}$). ^{15}N -ammonium tracer additions were generally kept close to 10% of ambient ammonium concentration.

Incubations were done using an *in situ* mooring line deployed at sunrise and recovered at sunset (~ 7 h later). Once recovered,

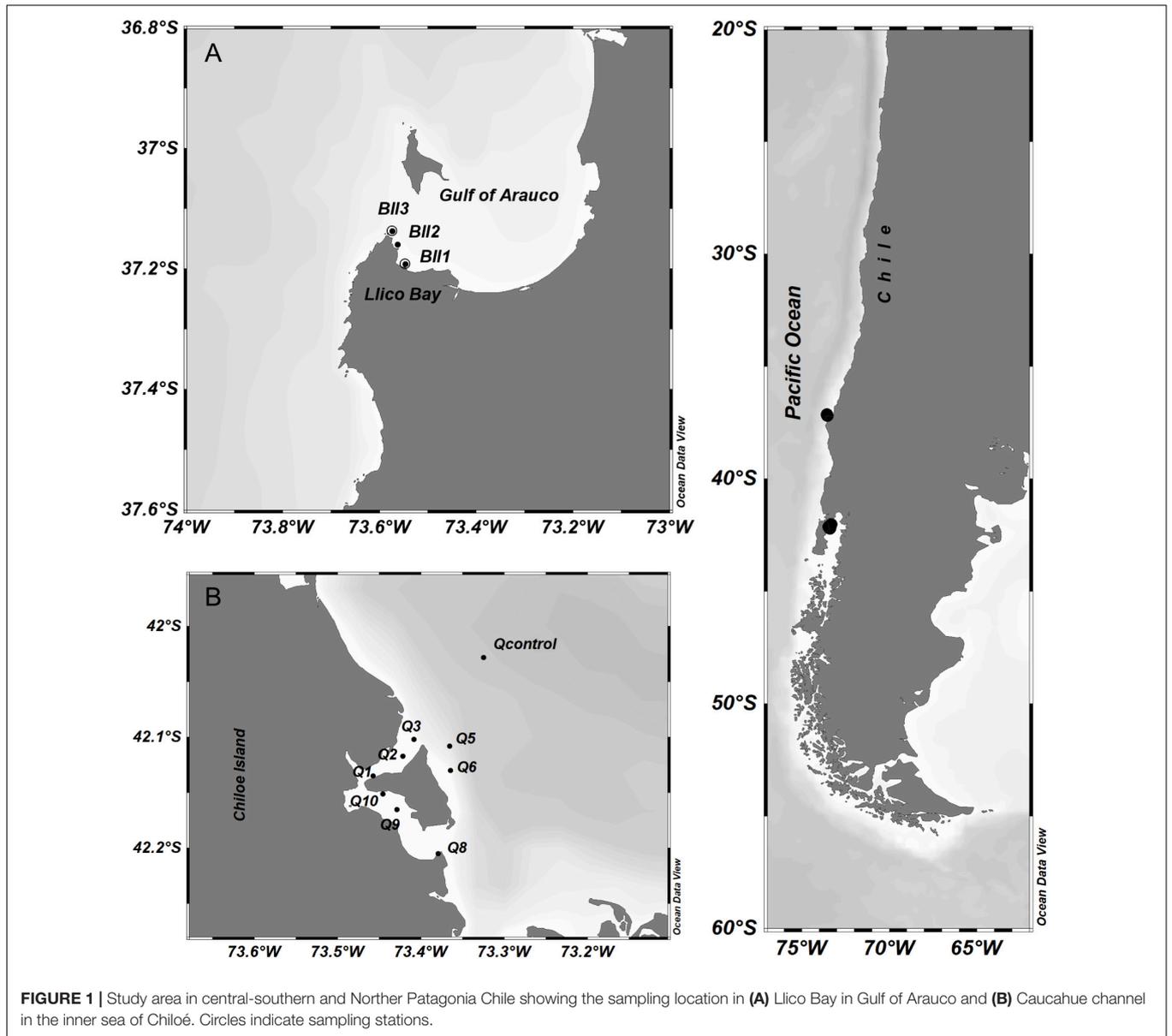


TABLE 1 | Geographical location and seawater sampling depths for hydrographic characterization at Llico Bay and Caucahue channel stations.

Location	Station	Latitude (°S)	Longitude (°W)	Sampling depth
Llico	BII1	37.192	73.547	2*, 4*, 6
	BII2	37.159	73.563	2*, 10*, 15
	BII3	37.137	73.574	2*, 10*, 20
Caucahue	Q2	42.177	73.422	2*, 10*, 20, 30*, 40
	Q3	42.102	73.409	2*, 10*, 20, 30*, 50
	Q5	42.108	73.366	2*, 10*, 20, 30*, 50, 60
	Q6	42.130	73.365	2*, 10*, 20, 30*
	Q9	42.165	73.429	2*, 10*, 20, 30*, 50
	Qc	42.028	73.325	2*, 10*, 20, 30*, 50, 65, 80
	Q10	42.165	73.429	2*, 10*, 20, 30*, 50

Incubations and seawater sampling used for in situ ammonium uptake experiments are in asterisk ().*

the bottles were filtered through 0.7 μm GF/F filters (Whatman; pre-combusted at 540°C, 4 h) using a vacuum pump. Filters were stored at -20°C until analysis by isotope mass spectrometry at the Laboratory of Biogeochemistry and Applied Stable Isotopes (LABASI) of Pontificia Universidad Católica de Chile using a Thermo Delta V Advantage IRMS coupled with Flash2000 Elemental Analyzer.

Deltamethrin and azamethiphos were added to primary production incubations at doses determined according to the concentrations used in sea lice treatments: 3 $\mu\text{g L}^{-1}$ for deltamethrin (Siwicki et al., 2010) and 200 $\mu\text{g L}^{-1}$ for azamethiphos (Davies, 2001; Canty et al., 2007; Burrige et al., 2010). Stock solutions of azamethiphos (Dr. Ehrenstorfer GmbH, 98.5% purity) and deltamethrin (Dr. Ehrenstorfer GmbH, 99.5% purity) were prepared by dissolving the reagents in dimethylsulphoxide (DMSO, MERCK, 99.9% analytical grade). The concentration of the pesticide solution added was 1.2 and 3 $\mu\text{mol L}^{-1}$ for azamethiphos and deltamethrin, respectively, as described in the **Table 2**. Bottles without pesticides were incubated as controls ($^{15}\text{NH}_4$; **Table 2**).

Seawater sampling and incubation depths at the sampling points were set between surface and the compensation depth (which in this case coincided with app ~ 2.7 times the depth of view of the Secchi disk). At this level the light availability allows phytoplankton growth and the oxygen produced is equal to the consumed by respiration. Incubation and sampling depths for Llico Bay were at 2 and 4 m (Bll1) and 2 and 10 m depth (Bll2 and Bll3). At Caucahue Channel the depths sampling and for incubation were 2, 10 and 30 m depth (**Table 1**). More details of treatments and controls for *in situ* ammonium uptake experiments were described in **Table 2**.

On-Deck Ammonium Uptake Experiments

To study ammonium photo and chemoautotrophic uptake, four on-deck experiments were performed using dual stable isotopes techniques ($^{13}\text{C}/^{15}\text{NH}_4$). Results of Carbon-13 tracer were published in Rain-Franco et al. (2018). On-deck experiments were developed at Llico Bay (Bll2 station) with surface waters (2 m), during winter and spring 2015, and summer and autumn 2016.

In order to simulate solar radiation, we performed atmospheric measurements of incident Photosynthetically Active Radiation (PAR) using a portable radiometer (RM-21 Dr. Gröbel, Germany) and these results are available in Rain-Franco et al. (2018). Photoautotrophic samples were incubated under a combination of two cut off filters to simulate the irradiance at the sampling depths: 0.3 Neutral density 209 (47% of average transmittance between 400 and 700 nm, Lee filters) and Steel Blue 117 (Lee filters). Dark treatments to obtain chemoautotrophic ammonium uptake were put in a closed incubator. Incubations were done in Duran Schott bottles (580 mL) and amended with a $^{15}\text{NH}_4$ solution (ICON isotopes IN 5037; 99% at 0.5 $\mu\text{mol mL}^{-1}$; final concentration in the sample in the 83 nmol L^{-1}) and pesticide solution as described in **Table 3**. The pesticide treatments used were azamethiphos

($^{15}\text{NH}_4 + \text{Az}$), deltamethrin ($^{15}\text{NH}_4 + \text{Dm}$) and emamectin benzoate ($^{15}\text{NH}_4 + \text{Em}$). The dose of emamectin benzoate (0.66 mmol L^{-1} , Dr. Ehrenstorfer GmbH) was estimated in order to obtain the highest possible experimental concentration by dividing the standard into two equal parts. Also, combined treatments were evaluated, azamethiphos-deltamethrin ($^{15}\text{NH}_4 + \text{Az} + \text{Dm}$) and azamethiphos-deltamethrin-emamectin benzoate ($^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$). All the incubations (light and dark) were done in duplicate for 6 h, with a subsample at 2 h. Bottles were incubated in 3 large aquariums of 50 L each, which were interconnected and received surface seawater with a continuous flow to maintain *in situ* temperature conditions (ca. 13°C). More details of treatments and controls of on-deck incubation are available at **Table 3**.

Data Analysis

Ammonium uptake rates were estimated as described in previous studies (Fernández et al., 2009; Fernandez and Farías, 2012) following Eq. (1):

$$\rho DI^{15}N = \left[\%R_{PON} - 0.36912 \right] * \left(PON * \frac{1000 * V_f}{14} \right) / \%R_{DIN} \quad (1)$$

where V_f is the filtrated volume, PON represents the amount of particulate N obtained by mass spectrometry (μg) and $\%R_{PON}$ is the enrichment in $^{15}\text{NH}_4$ in the GF/F filter after the incubation. $\%R_{DIN}$ is the excess of enrichment of ^{15}N after the inoculation (T_0) computed according to Eq. (2):

$$\%R_{DIN} = \left(\frac{^{15}N * ^{15}DIN}{V_b} + (DIN_i * 0.0036912) \right) / \left(DIN_i - \frac{^{15}N * ^{15}DIN}{V_b} \right) \quad (2)$$

where ^{15}N is the volume of isotopic solution ($^{15}\text{NH}_4$), ^{15}DIN represents the concentration of the ^{15}N added inoculums. The term 0.0036912 represents the natural abundance (average) of ^{15}N . DIN_i represents the concentration of DIN in the sample before the addition of tracer. V_b represent the incubation bottle volume. For *in situ* incubations, hourly rates were multiplied by 12 in order to obtain daily ammonium fixation values. Initial ammonium concentrations were taken from a nutrient profile prior to incubation and subsamples for final ammonium concentrations were taken directly from the incubation bottle before filtration.

For Llico Bay and Caucahue Channel *in situ* incubations a paired *t*-test was done to compare treatments with their respective control conditions. For on-deck incubations (Llico Bay), a two-way ANOVA was computed to evaluate significant variations among treatments and between incubation times after checking for normality assumptions (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). Whenever data did not pass the test, a log transformation was implemented. Pairwise multiple comparison was performed using a Tukey test as a *posteriori* analysis. All the statistical analyses were performed using the statistical software R¹.

¹<https://www.r-project.org/>

TABLE 2 | Summary of *in situ* ammonium assimilation experiment setup during Llico and Caucahue campaigns.

Location	Dates	Season	Station	Isotopes and pesticide solution concentration
Caucahue	18-30/06/2014	Winter	Qc, Q2, Q5, Q6, Q9	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$)
Caucahue	20-30/01/2015	Summer	Qc, Q2, Q3, Q5, Q9	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$)
Llico	21-25/07/2015	Winter	BLL1, BLL2, BLL3	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$)
Llico	21-25/10/2015	Spring	BLL1, BLL2, BLL3	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$)
Llico	05-01/01/2016	Summer	BLL1, BLL2, BLL3	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$)
Llico	21-29/04/2016	Autumn	BLL1, BLL2, BLL3	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$)

Az, Azamethiphos and Dm, Deltamethrin.

TABLE 3 | Summary of on-deck ammonium uptake incubations in Llico campaigns.

Location	Dates	Season	Station	Isotopes and pesticide solution concentration
Llico	21-25/07/2015	Winter	BLL2	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$)
Llico	21-25/10/2015	Spring	BLL2	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$)
Llico	05-01/01/2016	Summer	BLL2	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$)
Llico	21-29/04/2016	Autumn	BLL2	$^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) $^{15}\text{NH}_4$ (0.083 $\mu\text{mol L}^{-1}$) + Az (1.2 $\mu\text{mol L}^{-1}$) + Dm (3 $\mu\text{mol L}^{-1}$) + Em (600 $\mu\text{mol L}^{-1}$)

Az, Azamethiphos, Dm, Deltamethrin and Em, emamectin benzoate.

RESULTS

Chemical and Biological Seasonal Variability in Llico Bay and Caucahue Channel

Temperature and salinity in Llico Bay varied between seasons, showed a marked thermal stratification during spring 2015 and

summer 2016 in Bll1 and Bll2 stations, the maximum surface values was nearly 17°C at Bll1 station in summer 2016 and the maximum salinity values was ~ 35 in summer 2016 for the three stations (**Supplementary Figure 1**). Whereas in winter and autumn lower temperature values was observed (~ 12°C in winter) and as well lower salinity values (~ 32°C in winter 2015). Ammonium showed higher concentrations in autumn 2016 and spring 2015 compared with winter 2015 and summer 2016 at Bll1

and Bll2 stations. Station Bll3 showed the highest ammonium concentration and reached $9 \mu\text{mol L}^{-1}$ at 20 m depth in autumn 2016 (Figures 2A,E,I). Nitrate concentration showed a marked seasonal variability with higher concentrations in spring 2015 followed by winter 2015. The lowest nitrate concentration was observed in summer 2016 in almost all depths (Figures 2B,F,J). Nitrite was higher in winter 2015 at all stations (Figures 2C,G,K). The N/P ratio varied seasonally, although maximum values were observed in spring and winter 2015, particularly Bll3 station showed an average ratio of 25 (Figures 2D,H,L).

The highest concentrations of Chl-a were observed in summer 2016 in the three stations (Figure 2), and the maximum value was observed in surface at Bll3 station ($\sim 23 \text{ mg m}^{-3}$; Figure 2O). The lowest concentration was observed in autumn 2016 at Bll1, Bll2, and Bll3, at all depths. The highest bacterioplankton abundance was observed in spring 2015, with the maximum abundance at station Bll3 ($3,990 \times 10^3 \text{ cell mL}^{-1}$). Minor changes were observed in winter 2015, summer 2016 and autumn 2016 (Supplementary Figure 2).

A seasonal variability was also observed at Caucahue channel stations, lower temperature was observed during winter ($\sim 11^\circ\text{C}$) compared with summer ($\sim 16^\circ\text{C}$; Supplementary Figure 3). Salinity showed a strong vertical variation in winter, particularly at the station Q6. Ammonium concentration was higher in winter compared to summer in Caucahue Channel (Figures 3A,F), specifically at station Q2 ($5 \mu\text{mol L}^{-1}$) in surface waters, followed by station Q6 with $3 \mu\text{mol L}^{-1}$ at 20 m depth. The lowest concentration was found at the control station (Qc) with values close to zero throughout the water column (Figure 3A). In summer, the concentrations were generally below $0.3 \mu\text{mol L}^{-1}$ for all stations, with the exception of Q2 station ($>1 \mu\text{mol L}^{-1}$; Figure 3F). As well of summer, in winter, ammonium concentrations at Qc station were nearly zero throughout the water column.

Nitrate concentrations in winter and summer were in general below $3 \mu\text{mol L}^{-1}$, except at station Q1 and Q6 (Figures 3B–G), with maximum concentration of $11 \mu\text{mol L}^{-1}$ at 30 m depth and during summer, station Q5 showed maximum concentration of $11.5 \mu\text{mol L}^{-1}$ at 20 m. Nitrite concentration were in general higher in winter compared to summer (Figures 3C–H). During winter, nitrite concentration tends to increase with the depth, the highest values was found at station Q6 ($2 \mu\text{mol L}^{-1}$). In summer, the concentration was lower than $0.5 \mu\text{mol L}^{-1}$. The N/P ratio varied greatly depending on stations and depths. Station Qc had the lowest ratios, 1.71 ± 1.17 in winter and 2.84 ± 1.14 in summer (Figures 3D–I). The highest ratio was observed at station Q5 in winter 22.8 at 2 m.

Chl-a concentration was higher in summer at the station Qc and between stations Q3 and Q2, nearly 4 mg m^{-3} (Figures 3E–J). Concentration in winter were lower than 1 mg m^{-3} for all stations. Bacterioplankton in Caucahue Channel showed a marked seasonal influence. In winter showed abundances under the $500 \times 10^3 \text{ cell mL}^{-1}$ and during summer in general the abundances were over the $500 \times 10^3 \text{ cell mL}^{-1}$ (Supplementary Figures 2E–F). In winter bacterioplankton was more abundant at station Q9 and lowest at station Q5. In summer, maximum abundances were observed at stations Q9 and the

lowest abundances were observed in general at control station (Supplementary Information; Figure 2B).

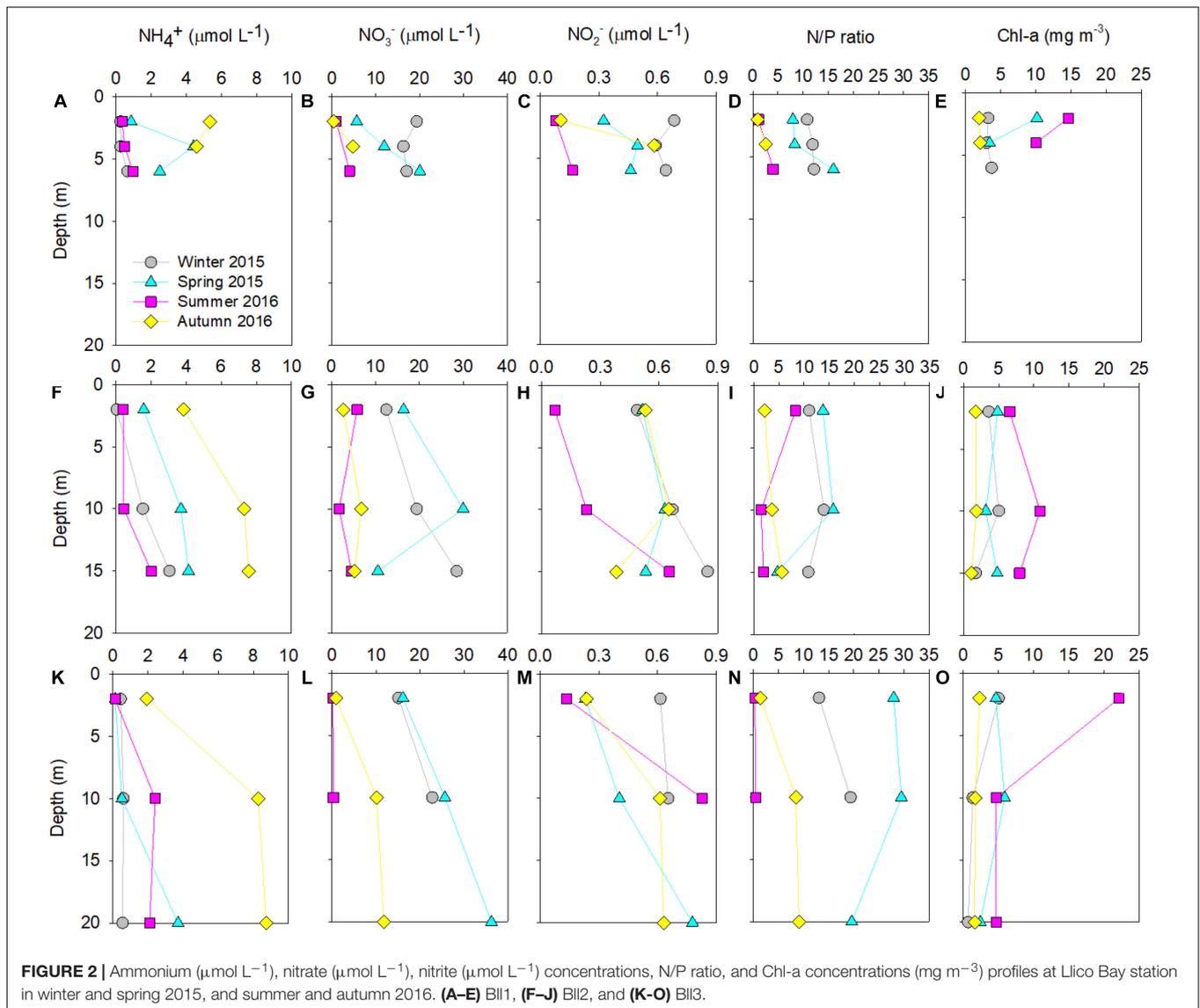
In situ Ammonium Uptake Rates at Llico Bay and Caucahue Channel

Ammonium uptake rates were higher in surface waters compared to deeper levels (10 m) at all stations and seasons (Figure 4). At station Bll1 assimilation rates observed in spring 2015, were 93 and 95% higher than winter 2015 and autumn 2016 (7.9 and $10.4 \mu\text{mol L}^{-1}\text{d}^{-1}$ at 2 and 4 m depth, respectively; Figures 4A–C). Station Bll2 showed the highest ρNH_4^+ values during autumn 2016 reaching $3 \mu\text{mol L}^{-1}\text{d}^{-1}$ at 2 m and the minimum rate was observed in spring at 10 m depth ($0.5 \mu\text{mol L}^{-1}\text{d}^{-1}$). The same pattern was observed in Bll3 with higher ammonium uptake during autumn 2016 at 2 m depth ($2.5 \mu\text{mol L}^{-1}\text{d}^{-1}$) and a decrease in 81% at 10 m depth. The lowest rate was observed in winter 2015 at 10 m depth ($0.9 \mu\text{mol L}^{-1}\text{d}^{-1}$; Figures 4H–J).

In treatments where pesticides were added, ammonium uptake showed variable responses compared to the control ($^{15}\text{NH}_4$; Table 2). The addition of pesticides caused an increase in ammonium assimilation in winter 2015 (Figures 4A,D,H) compared to controls, particularly at station Bll3 at 10 m depth, in which rates in all treatments ($^{15}\text{NH}_4 + \text{Az}$, $^{15}\text{NH}_4 + \text{Dm}$, and $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$) increased by 42% compared to their controls. At Bll1 and Bll2 the rates increased between 2 and 15% compared to the controls ($^{15}\text{NH}_4$), except for the combined treatment $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$ at Bll1 station and $^{15}\text{NH}_4 + \text{Az}$ at 10 m at station Bll2.

In general, the addition of pesticides in autumn 2016 resulted in a decrease of ammonium utilization rates compared to the controls. At Bll1 station rates were 20% lower than the control ($^{15}\text{NH}_4$) for the treatment $^{15}\text{NH}_4 + \text{Dm}$ (4 m; Figure 4C). At Bll2 station the rates decreased between 3 and 18%, in the treatment with deltamethrin and in the combined treatment $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$ at 2 m, respectively. However, at this station was also observed an increase in a 55% the ρNH_4^+ values for $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$ treatment at 10 m depth. In the station Bll3, an inhibition was only observed in the treatments with a single pesticide ($^{15}\text{NH}_4 + \text{Az}$ and $^{15}\text{NH}_4 + \text{Dm}$) at 2 m depth. In subsurface layer (10 m) the addition of pesticides caused an increase in the ammonium assimilation rates up to 132% compared to the control for the treatment $^{15}\text{NH}_4 + \text{Az}$. This increase was also observed for $^{15}\text{NH}_4 + \text{Dm}$ (89%) and $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$ (46%; Figure 4J). However, all these differences reported were non-significant (t -test > 0.05) for pesticide additions at any station during winter 2015 and autumn 2016. For spring 2015 and summer 2016 no data are available for treatments with pesticides (Figure 4).

In Caucahue channel, ammonium uptake varied throughout the stations and also between seasons and depths. The highest ρNH_4^+ values were observed at station Q2 in winter ($0.5 \mu\text{mol L}^{-1} \text{d}^{-1}$) and in summer ($0.6 \mu\text{mol L}^{-1} \text{d}^{-1}$) in surface layer (Figures 5, 6, respectively) inside of the area influenced by the farming centers, and the lowest rates was observed at station Q9 in both season (Figures 5E, 6E). In summer 2015, the chemoautotrophic ammonium utilization (dark treatment)



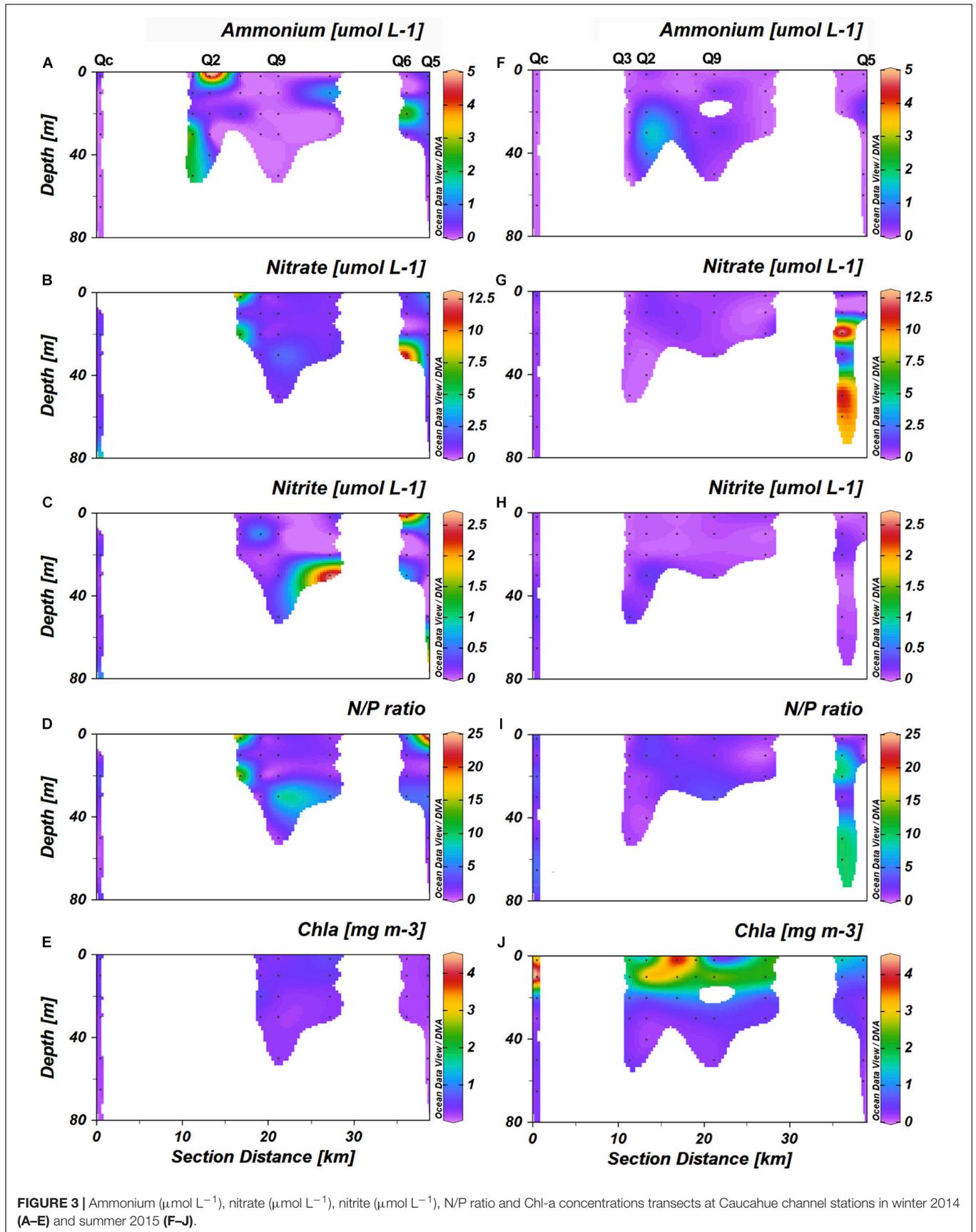
was 10 times higher than the photoautotrophic nitrogen uptake in surface at Q9, but in general the both photo and chemoautotrophic are in the same range of values.

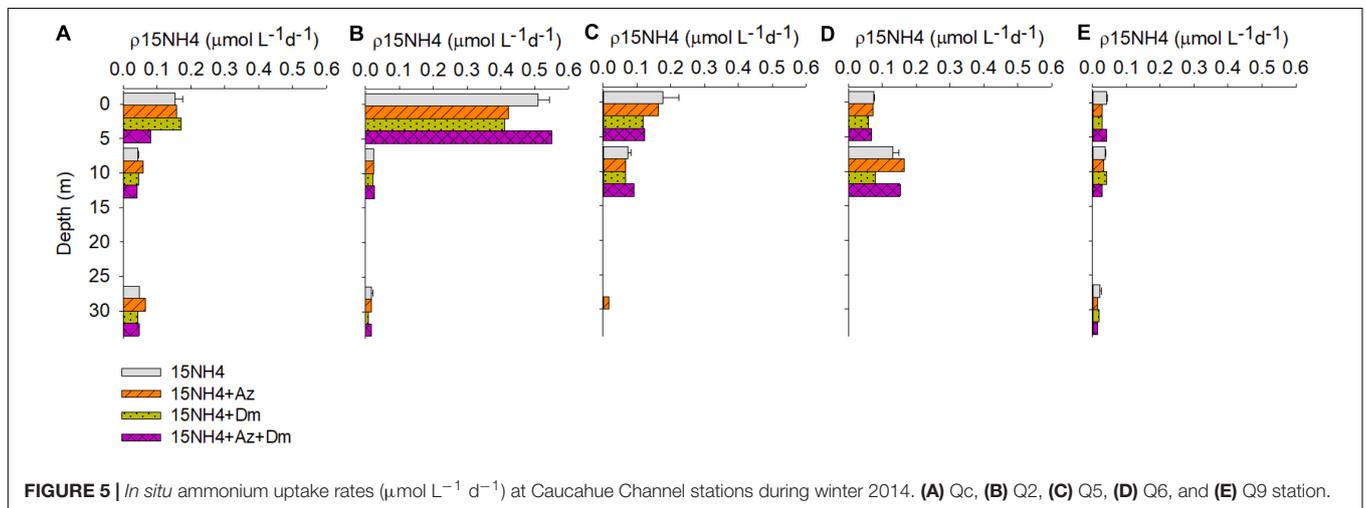
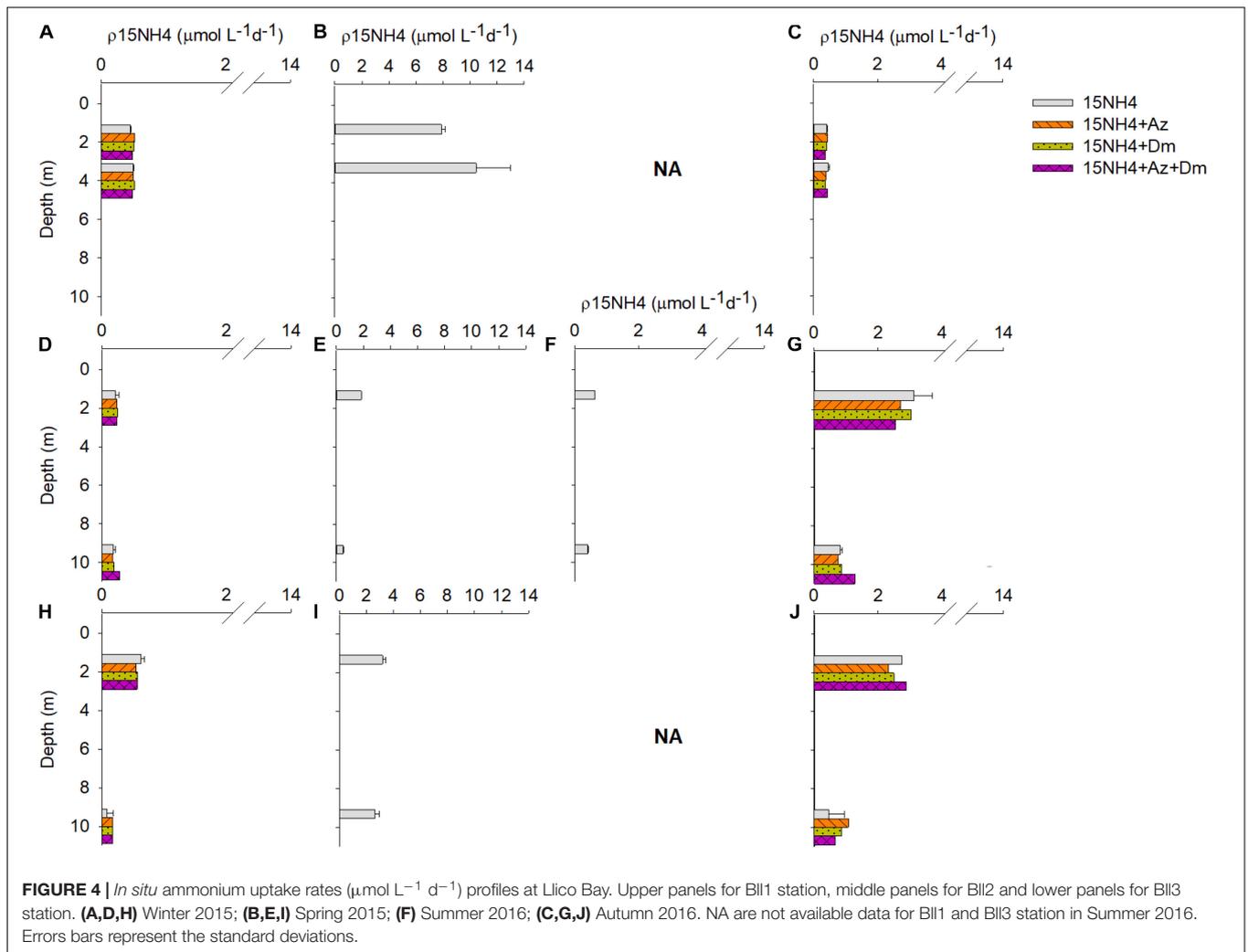
In winter 2014 (Figure 5), pesticide additions resulted in an increase in ammonium uptake in the treatment $^{15}\text{NH}_4 + \text{Az}$ at station Qc between 3% (2 m) and 40% (30 m). In general, deltamethrin addition resulted in a decrease in ammonium assimilation rates, the highest difference found with the control ($^{15}\text{NH}_4$) was at station Q2 (Figure 5B). At this station which was located close to the farms, ρNH_4^+ decreased by 53% (30 m depth), followed by a reduction of 33% at the station Q5 in surface (Figure 5C). However, deltamethrin addition cause an increase in the rates of ammonium assimilation in the first 10 m depth at station Qc, between 7 and 12% higher than the controls (Figure 5A).

The combined treatment $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$ also resulted in a variable response to the pesticide addition (Figure 5). Qc station

showed a decrease in the ammonium assimilation rates up to 47% at the surface. This pattern was also observed at station Q9, observed rates up to 29% less than the controls. Higher rates compared to the control was observed at Q2, up to 13% higher at 10 m depth. At the same depth, an increase was observed at stations Q5 (13%) and Q6 (23%). However, these values were not significantly different from the control treatment ($^{15}\text{NH}_4$; paired *t*-test, $p < 0.05$).

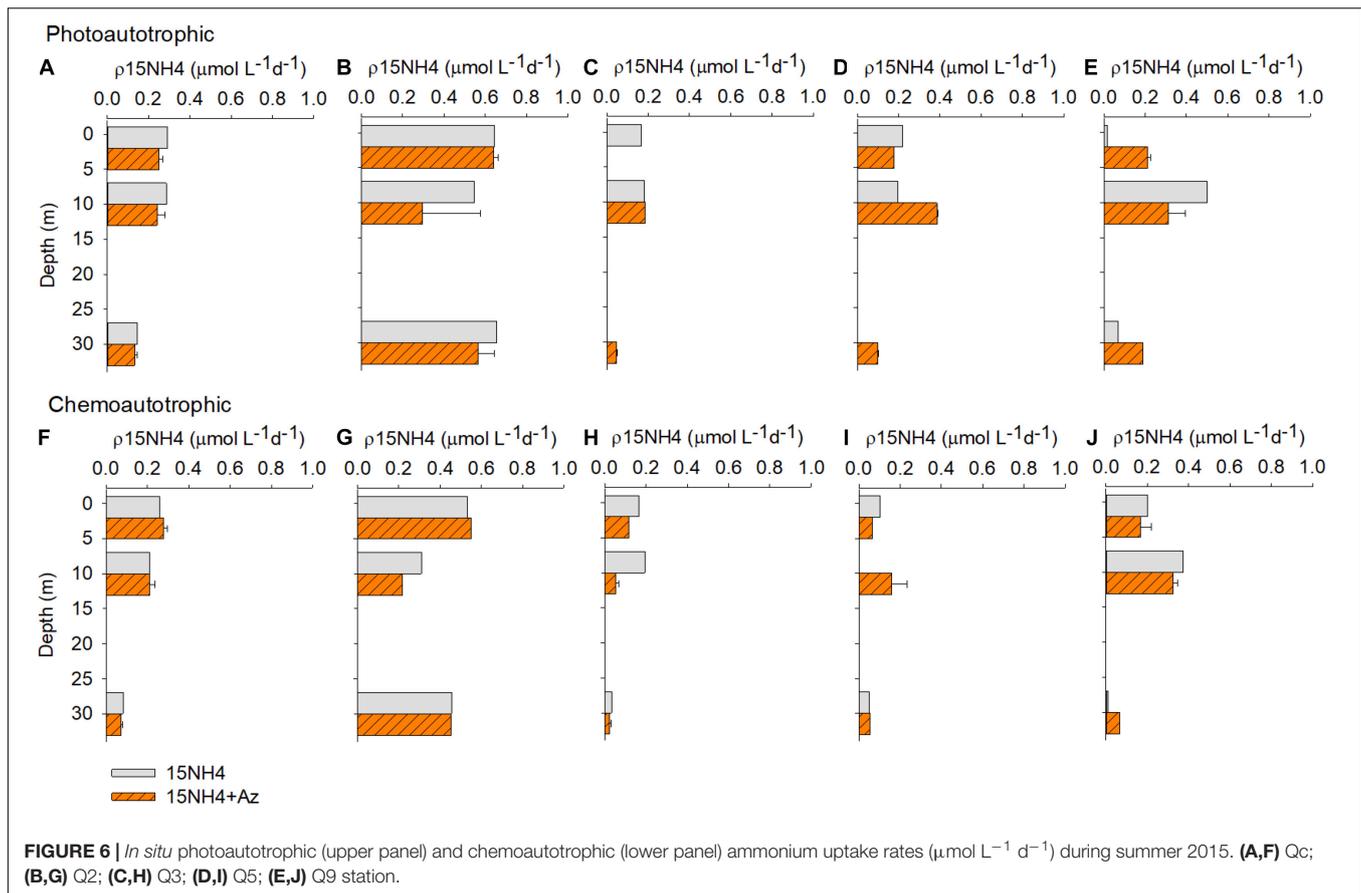
In summer (Figure 6), the *in situ* incubations were made under light (photoautotrophic) and dark conditions (chemoautotrophic). At station Qc, the addition of azamethiphos resulted in a significant decrease in ammonium uptake under light conditions (in average 13%; paired *t*-test > 0.05). However, we also observed an increase in ρNH_4^+ values at 2 and 30 m in the station Q9, being up to 100 times higher the rates obtained after the addition of azamethiphos. However, these increases were not significant, since they were not observed for





all depths (paired *t*-test > 0.05). The chemoautotrophic uptake showed a decrease though the water column only at station Q3 between 23 and 72% lower than the controls. Slight increase

was observed for the chemoautotrophic ammonium utilization (**Figures 6F,G,I,J**) after the addition of azamethiphos, except for station Q9 in which we observed rates 570% higher than the



control (Figure 6). But, these effects were not significant (paired t -test > 0.05).

Pesticide Effect on Surface Ammonium Uptake Under Light and Dark Conditions: On-Deck Experiments

On-deck ammonium uptake experiments (Figure 7) showed higher photoautotrophic control rates ($^{15}\text{NH}_4$) in autumn 2016 ($0.14 \mu\text{mol L}^{-1} \text{h}^{-1}$) after 2 h of incubation followed by spring 2015 ($0.1 \mu\text{mol L}^{-1} \text{h}^{-1}$) at the same incubation time (Figures 7A,B). On the other hand, the highest chemoautotrophic ammonium uptake rate was observed in spring 2015 ($0.1 \mu\text{mol L}^{-1} \text{h}^{-1}$). This rate was equivalent to 100% of photoautotrophic ammonium assimilation after 2 h of incubation, and after 6 h of incubation the chemoautotrophic ammonium uptake was 7% higher than photoautotrophic ammonium assimilation (Figures 7E–H). This was also observed in summer 2016 when the chemoautotrophic ammonium uptake was equivalent to an 85% of the photoautotrophic at 2 h of incubation.

Results from a two-way ANOVA applied to light condition experiments showed significant differences in all the experiments between the treatments with pesticides and/or the time (Table 4). In winter 2015, the treatment $^{15}\text{NH}_4 + \text{Az}$ and $^{15}\text{NH}_4 + \text{Dm}$ were significantly different from the control ($^{15}\text{NH}_4$). The $^{15}\text{NH}_4 + \text{Az}$ was 32% lower after 2 h of incubation and dropped

by 21% after 6 h of incubation compared to their control values ($p = 0.001$). The treatment $^{15}\text{NH}_4 + \text{Dm}$ was also a 53 and 64% lower after 2 and 6 h of incubation, respectively ($p = 0.001$). Photoautotrophic ρNH_4^+ values were significantly lower at the end of the incubation (6 h) compared to the first 2 h of incubation for control and treatments with pesticides ($^{15}\text{NH}_4$:30%; $^{15}\text{NH}_4 + \text{Az}$:16%; $^{15}\text{NH}_4 + \text{Em}$:47%; $^{15}\text{NH}_4 + \text{Dm}$: 6% lower at 6 h of incubation; Table 4). In spring 2015, the treatment $^{15}\text{NH}_4 + \text{Em}$ and the combined pesticides treatment ($^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$) were significantly different from their controls ($p = 0.001$), showing a decrease in ammonium assimilation rates. The $^{15}\text{NH}_4 + \text{Em}$ treatment rates were 77 and 23% lower than their control after 2 and 6 h of incubation, respectively. The combined treatment ($^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$) was 39 and 23% lower compared to their controls at 2 and 6 h, respectively. Photoautotrophic ammonium uptake rates during spring 2015 were significantly lower ($p = 0.001$) after 6 h of incubation (69% average) in control and in the treatments amended with $^{15}\text{NH}_4 + \text{Az}$, $^{15}\text{NH}_4 + \text{Bm}$, $^{15}\text{NH}_4 + \text{Em}$, $^{15}\text{NH}_4 + \text{Az} + \text{Dm}$, $^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$. Light treatments during summer 2016 did not show significant differences between the treatments amended with pesticides and controls ($p = 0.469$) but values over time were significantly different from each other. After 6 h of incubation the light treatments were in average 58% lower than values obtained during the first 2 h of incubation. The same result was found in autumn 2016 when treatments did

not show significant differences with pesticides compared to the controls but assimilation rates were significantly lower (average 59%) at 6 h compared to the first 2 h of incubation.

A two-way ANOVA for dark incubation (Table 4) showed that ammonium uptake in spring 2015 was 36 and 30% lower in the treatments $^{15}\text{NH}_4 + \text{Em}$ and $^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$ compared to the control ($^{15}\text{NH}_4$) at 2 and 6 h of incubation, respectively. The combined treatment $^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$ was a 42 and 25% lower than the control after 2 and 6 h of incubation ($p = 0.001$). These differences were also found between time ($p = 0.001$) and also the interaction of the pesticides and time ($p = 0.013$). In average, dark ammonium utilization was 56% lower after 6 h of incubation than the first 2 h in spring 2015. In winter 2015, did not find significant differences in the chemoautotrophic ammonium assimilation between control and the treatments amended with pesticides ($p = 0.6$) and between the incubation times ($p = 0.06$). On-deck experiments in summer and autumn 2016 in dark conditions did not show differences between the treatments with pesticides and its controls ($p = 0.97$ and $p = 0.94$, respectively). However, values were significantly higher at 2 compared to 6 h of incubation by 59% in summer and by 69% in autumn 2016.

DISCUSSION

Studies oriented specifically on the impacts of anti-lice pesticides to microbial communities involved in primary production are quite limited (Knapp et al., 2005; Burrige et al., 2010; Rain-Franco et al., 2018). Our results complement a first report of the effects of pesticides on rates of carbon assimilation (Rain-Franco et al., 2018) for the same study area, and as part of the same experiment. Overall the results provide new evidence that anti-lice pesticides can affect fundamental processes of marine ecosystem functioning.

Here we report ammonium uptake rates at Llico Bay and Cauahue Channel. In central-southern Chile, ammonium utilization in spring-summer season is high ($0.23 \pm 0.4 \mu\text{mol L}^{-1}\text{d}^{-1}$ on average; Fernandez and Fariás, 2012). At Llico Bay, ammonium was always present in subsurface waters at relatively high concentrations (Figures 2A,E,I), and the rates of ammonium assimilation observed in subsurface waters suggest that the entire surface pool of ammonium cannot be taken within 24 h. This pattern, was also observed in Cauahue Channel, except at control station (Qc), outside of the influence of farms, in which the ammonium concentration was very lower in surface waters, suggesting that the ammonium pool could be used within 24 h. Inside the channel is expected to find higher accumulation of organic matter due to the presence of salmon farms and consequently higher remineralization rates that can explain the higher ammonium concentration found in the channel (Olsen et al., 2014; e.g., Q2) and this is contrasted with the Qc station in which concentration was nearly zero.

Ammonium is highly bioavailable and is often the preferred N compound assimilated by bacterioplankton and phytoplankton (Glibert et al., 2016). During dark conditions, ammonium uptake was higher than the photoautotrophic uptake, being up to

10 times higher at station Q9 in Cauahue channel (summer 2015). This was also found during on-deck experiments in Llico, in which the chemoautotrophic ammonium uptake was equivalent to 100% of the photoautotrophic uptake in spring 2015. Dark N uptake has been reported that can be anywhere from 10 to 100% of the uptake rate measured in the light, and this varies with physiological conditions of the populations and the species (Mulholland and Lomas, 2008). Nevertheless, these results suggest a potentially important contribution to regenerated production in these areas, affecting surface N dynamics, modifying the balance between new and regenerated production and consequently the downward flux of N.

The impact of pesticide in ammonium uptake was highly variable between the different pesticides amendments, significant effects were detected for a single pesticide (azamethiphos) during *in situ* incubations and for combined pesticides (azamethiphos-deltamethrin-emamectin benzoate) during on-deck experiments. The response of ammonium utilization to the addition of azamethiphos revealed an inhibition of the photoautotrophic uptake at station control (Qc) in Cauahue Channel in summer 2015. These findings are in contrast to the response obtained in C uptake, in which the addition of azamethiphos triggers an increase in the phototrophic C utilization (Rain-Franco et al., 2018), and also, are in contrast with the findings reported by Burrige et al. (2010) that suggest neutral effects on primary production, since no changes in dissolved oxygen and chlorophyll-a concentrations were observed in field studies in Canada.

Azamethiphos is an organophosphate insecticide highly soluble in water (Canty et al., 2007; Burrige et al., 2010). A very recent study, showed higher microbial degradation capacity of the pesticide azamethiphos nearly to our study area in Cauahue channel, suggesting that microorganism can use organophosphate pesticides as a source of C and phosphorus for their metabolism (Garcés et al., 2020). Periods of N and phosphorus limitation have been reported for the inner Sea of Chiloé (Iriarte et al., 2007; Olsen et al., 2014, 2017), in our results, we observed a higher increase in ammonium assimilation in surface and deeper waters at station Q9 (summer) in Cauahue Channel. This station showed a lower concentration of N and phosphorus (Figure 3) and a high bacterioplankton abundance, so the addition of this organic phosphorus compounds should be used as a nutrient source. This also can occur in the limit of the photic zone in which the competition of nutrients increases, and pesticides, such as azamethiphos, can be used by the microbial communities. Such increases in phototrophic and also chemoautotrophic ammonium uptake were seen during winter at stations Q5, and Q2 (Figure 5), and at station Bll3 in Llico Bay (Figure 4).

On the other hand, emamectin benzoate produces a decrease in photo and chemoautotrophic ammonium utilization between a 22 and 77% as we observed in on-deck experiments at Llico bay. The emamectin benzoate is a semi-synthetic derivative of a chemical produced by the bacterium, *Streptomyces avermitilis* (Bravo et al., 2008). The effects of this pesticide on non-target organisms has been reported by a number of authors using, however, a few species (e.g., mussels, fishes, American lobster and copepods; Willis and Ling, 2003; Burrige et al., 2010, 2004).

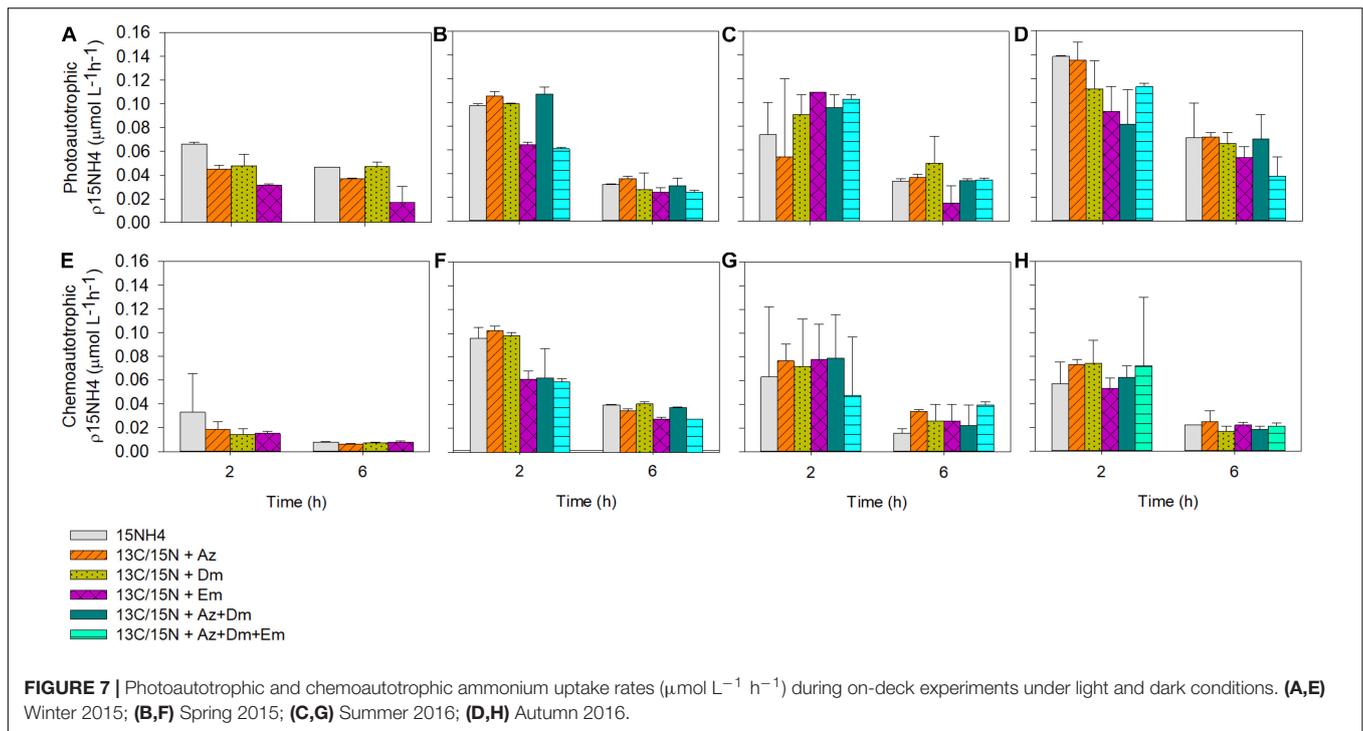


FIGURE 7 | Photoautotrophic and chemoautotrophic ammonium uptake rates ($\mu\text{mol L}^{-1} \text{h}^{-1}$) during on-deck experiments under light and dark conditions. **(A,E)** Winter 2015; **(B,F)** Spring 2015; **(C,G)** Summer 2016; **(D,H)** Autumn 2016.

TABLE 4 | Results from two-way ANOVA examining the effect of pesticides and the incubation time on N uptake rates obtained during on-deck experiments under light and dark conditions at Lico Bay.

Season	Date	Source of variation	d.f	Light		d.f	Dark	
				F	p		F	p
Winter	July 2015	Pesticides	3	19.701	0.001	3	0.579	0.644
		Time	1	12.196	0.008	1	4.678	0.063
		Pesticides × Time	3	1.815	0.222	3	0.503	0.691
		Residual	8			8		
Spring	October 2015	Pesticides	5	15.363	0.001	5	9.711	0.001
		Time	1	814.094	0.001	1	189.122	0.001
		Pesticides × Time	5	9.955	0.001	5	4.673	0.013
		Residual	12			12		
Summer	January 2016	Pesticides	5	0.985	0.469	5	0.154	0.974
		Time	1	25.101	0.001	1	11.881	0.005
		Pesticides × Time	5	1.118	0.405	5	0.336	0.881
		Residual	11			12		
Autumn	April 2016	Pesticides	5	2.643	0.078	5	0.232	0.941
		Time	1	49.706	0.001	1	32.189	0.001
		Pesticides × Time	5	1.734	0.201	5	0.263	0.924
		Residual	12			12		

Significant values are presented in bold.

Our results are in accordance with the results found in C uptake, suggesting that emamectin benzoate can potentially act as a depressor of C fixation (Rain-Franco et al., 2018) and now also for ammonium assimilation. The inhibition in ρNH_4^+ values was also found in the combination of the three pesticides treatment ($^{15}\text{NH}_4 + \text{Az} + \text{Dm} + \text{Em}$) in autumn. However, this effect was not detected in the combined treatment with azamethiphos and deltamethrin ($^{15}\text{NH}_4 + \text{Az} + \text{Dm}$), suggesting

that the inhibitory effect was related to the addition of emamectin benzoate. Previous studies suggest that toxicity can be increased using a combination of compounds compared to the use of single chemical (Laetz et al., 2009), but in our case the higher inhibitory response was found with a single addition of emamectin benzoate and not after a combined addition.

There are no studies dealing directly with deltamethrin and microorganism. From our data, deltamethrin produce an

inhibitory or toxic effect during winter 2015 reducing up to 64% the ammonium assimilation after 6 h of incubation. The toxic effects have been also observed in amphibians, crustacean and mollusk (Bhanu et al., 2011; Ernst et al., 2014). However, due to the high variability found in our results further studies are needed.

The interaction between the salmon cages, local hydrodynamics and local hydrographic characteristic are important factors to evaluate the dispersal propagation and the impact on non-target species of pesticides in the aquatic environment. Dispersion and dilution rates of the pesticides depend on the chemical nature (e.g., solubility, adsorption capacity, and chemistry composition) and the hydrographic characteristics of the area. A residual chemical compound concentration can persist in the area which is likely to impact non-target species (Nash, 2003). Uneaten food is point sources of emamectin benzoate to the surrounding ambient, and it is estimated that approximately 5–15% of the administered food is uneaten (Chen et al., 1999). Bloodworth et al. (2019) demonstrated a widespread detection of emamectin benzoate in sediments around Scottish fish farms. As well, Herrera et al. (2018) through a modeling methodology to assess the hydrodynamic effects of a salmon farm in a Patagonia channel, found that a considerable part of the enclosed volume within the cages of a fish farms remains circulating in the area of channels in Patagonia, suggesting an impact in the dispersal of pesticides in the area. The topical bath treatment method applied for azamethiphos will most likely result in rapid dilution of active ingredients. Azamethiphos has been detected up to 1000 m away from the location treatment (Ernst et al., 2014). The dispersion and dilution rates of the different anti-lice treatments utilized in this study can explain the high variability found in our results through the different stations and depths sampled, and this variability was also found in C assimilation (Rain-Franco et al., 2018). These results suggest a strong impact of the choice of pesticide used, the seasonal variability of the study area and the distance of salmon farming on the impact on microbial community and consequently on local primary productivity.

The impact of pesticides can be either positive by enhancing ammonium uptake or could be inhibitory for microorganism but either way it will impact the natural N budget and this impact was not only detected in photoautotrophic ammonium uptake; pesticides can also affect the chemoautotrophic communities, and potentially can modulate the regenerated primary production and therefore the balance between new and regenerated production and organic matter export. Taken together, our findings and those of Rain-Franco et al. (2018) show significant alterations in C and N assimilation.

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In conclusion, the use of anti-lice pesticides can produce changes in photo and chemoautotrophic ammonium uptake in central-southern and northern Patagonia in Chile. Azamethiphos act as depressor of ammonium assimilation but also can be used by phyto- and bacterioplankton under nutrient limitation conditions. Emamectin benzoate can act as a strong depressor as well for deltamethrin and these changes were strongest if a single pesticide is applied as opposed to a combination of two or more pesticides. We conclude that pesticides in marine waters have the potential for altering local primary production fluxes and therefore can impact biological ecosystem productivity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CF designed the experiments and lead the research project, carried out the sample collection, and experiments. VV-C analyzed the data and participated in data interpretation. VV-C and CF wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.602002/full#supplementary-material>

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Conflict of Interest: 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.

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