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Aquatic one health—the intersection of marine wildlife health, public health, and our oceans

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Aquatic one health—the intersection of marine wildlife health, public health, and our oceans

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Editorial: Aquatic one health — the intersection of marine wildlife health, public health, and our oceans

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Editorial on the Research Topic
Aquatic one health—the intersection of marine wildlife health, public health, and our oceans

Plastics, disease pathogens, overfishing, and climate change are major threats to marine animal health. The scientific, public health, and veterinary medical communities are crucial in addressing these threats. A One Health approach, in which various disciplines are integrated together to promote the protection and preservation of human, animal, and environmental health, represents an ideal model to address marine health issues. One Health initiatives are becoming more popular globally (Delesalle et al., 2022); however, most focus on human health, and less on animal health, with very few addressing environmental health. Although the oceans and their ecosystems cover most of the planet, playing a pivotal role in the health and welfare of humans and animals, relatively little research has been published on One Health within aquatic ecosystems (Selbach et al., 2022). This Research Topic highlights how the health of humans, marine wildlife, and the ocean environment are holistically integrated through a One Health framework. The articles cover multiple marine ecosystem components such as fish, marine mammals, and invertebrates.

Pathogens potentially impacting marine wildlife, as well as the environment and humans, such as *Escherichia coli*, *Mucor* spp., and *Toxoplasma gondii*, are represented in the Research Topic. Anthropogenic sources of *E. coli* appear in terrestrial and freshwater ecosystems, but relatively less is known about *E. coli* diversity in marine ecosystems. In the article by Grunwald et al., a large diversity of sequence types (STs), associated with animals,

were found in an area of the Salish Sea, (Washington, USA) near to where farm animals are raised. Many of the STs identified have been associated with virulence in humans, while for others, no reference sequences could be identified.

Diversity of bacterial pathogens in gill microbiome of eastern Mediterranean wild fish was investigated by Itay et al.. Using a next generation sequencing approach on 16S amplicons collected from 89 individual fish, 177 unique values (*i.e.*, bacteria species) were identified. A total of 41 bacteria were known to have pathogenic potential to humans and/or marine animals. Even more interesting, is that a total of 36 bacteria had varying human clinical relevance or zoonotic potential. Finally, the literature also revealed that from 41 potentially pathogenic species, 14 bacteria were known marine animal pathogens, suggesting that some of those bacteria which are potentially pathogenic to humans, may also cause disease in marine wildlife.

Reports of disease due to the fungi *Mucor* spp., are increasing globally, and are considered a One Health concern in humans and animals. In humans, infections can occur in individuals who are immunocompromised, have elevated circulating serum iron, or uncontrolled diabetes; however, predisposing factors are less known in marine mammals. A qualitative risk assessment performed on a series of cases in harbor porpoises (*Phocoena phocoena*) in the inland waters of Washington State, USA, revealed elevated liver iron as a risk factor (Norman et al.). Another mycotic disease of growing One Health concern, lobomycosis-like disease (LLD), due to *Lacazia loboi*, causes a chronic and progressive dermatitis in humans and cetaceans, and is transmissible between the two (Reif et al., 2013). Endemic to central and south America, little is known about the epidemiology, pathology, and current expansion of LLD in the southwestern Gulf of Mexico (SWGM). An investigation of LLD in coastal SWGM bottlenose dolphins (*Tursiops truncatus*) revealed disease prevalence is relatively low, but habitat quality, and demographic and social characteristics of the dolphins may be influencing its geographical expansion into SWGM (Gálvez et al.). Furthermore, global and local climate variability may influence the epidemiology of LLD which could impact coastal human and dolphin health in the SWGM.

An atypical and rare genotype of the parasitic protozoan, *Toxoplasma gondii*, first isolated in Canadian cougars (*Puma concolor*), was detected for the first time in California sea otters (*Enhydra lutris nereis*). The resulting infection caused a new and intense lesion pattern of severe steatitis in otters. (Miller et al.). This parasite may be a One Health concern due to its high zoonotic potential and risk of infection from marine food sources shared by humans and otters.

Marine mammals have long been recognised as sentinels of marine ecosystem health, and Hart et al. adapted this approach to investigate the ubiquity of microplastic pollution risk for coastal communities that rely on seafood. Investigating gastric samples of common bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, FL (USA), they highlighted the animals' likely exposure to microplastics *via* contaminated prey, augmenting recent findings that concentrations of prevalent phthalate exposure in these dolphins was exceeding those of human reference populations. Monitoring the health status of marine mammals through systematic investigations of stranded animals in a One Health context is a strategy developed in metropolitan France (Wund et al.). The authors propose a common regional European strategy concerning analyses carried out together with other European Union Member States under the Marine Strategy Framework Directive of the European Union, using an integrated vision of public, animal and environmental health.

The Deepwater Horizon (DWH) oil spill of 2010 resulted in a high occurrence of chronic, moderate to severe, pulmonary disease in bottlenose dolphins living in Barataria Bay, Louisiana, USA, one of the most heavily-oiled estuaries. Aspiration of oil is the most likely exposure route, with alveolar interstitial syndrome being the most significant factor in the lung disease seen in these dolphins. Although physiologically adapted for swimming and diving, dolphin lungs are also susceptible to harmful effects of inhaled pollutants. They are air breathing mammals that live and feed along the coastline and thus should be considered sentinel species. The chronic and progressive lung disease found in dolphins inhabiting areas most affected by the DWH arguably provides insight into the long-term effects of contaminants, such as oil spills, on other animals (including humans) and the environment (Smith et al.).

Cultivated animals are globally traded, facilitating the spread of serious infectious diseases. Several trans-boundary aquatic animal diseases have swept through regions over the past 30 years, causing massive economic and social losses, through introduction, establishment, and spread of pathogens into new geographic areas. *Perkinsus* sp. dinoflagellates, internationally reportable pathogens of concern, are extremely virulent for clams and oysters. Perkinsosis, an important disease reported worldwide in bivalves and gastropods, can be responsible for large mortality events due to their extensive invasiveness and virulence. The first known report of *Perkinsus* sp. in Mediterranean mussels, *M. galloprovincialis*, is described in a mussel farm from the Campania region of Italy (Carella et al.).

The complex threats marine ecosystems face was revealed in the studies reported in this Research Topic. The studies highlighted threats to marine health that demand international cooperation and cross-disciplinary knowledge. A One Health framework provides an appropriate approach to handling the addressed marine threats in this Research Topic that require cross-disciplinary skills.

Author contributions

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

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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Risk factor determination and qualitative risk assessment of Mucormycosis in Harbor Porpoise, an emergent fungal disease in Salish Sea marine mammals

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Mucorales infections are increasing in frequency and are a One Health pathogen of concern. In humans and domestic animals, risk factors include being immunocompromised, elevated circulating serum iron, contaminated open wounds, or metabolic diseases such as ketoacidosis or uncontrolled diabetes. Mucormycosis was first identified in 2012 in Pacific Northwest marine mammals, predominantly in harbor porpoises. We performed an assessment to determine the overall qualitative risk, or risk score, of mucormycosis in harbor porpoises. Risk factors for this disease are unknown in aquatic mammals. In a separate risk factor analysis, potential risk factors such as pollutants, trace metals (e.g., iron), and co-infection with other pathogens (e.g., viruses and *Brucella* spp.) were examined in mucormycosis cases and noncases using a matched case-control study design, to determine the presence and strength of association of these factors with mucormycosis. Disease severity (gross and histopathology) and exposure scores were multiplied together to obtain the overall risk scores of 9 -16 which corresponded to moderate and severe, respectively. In the risk factor analysis, the factors most strongly associated with a mucormycosis case, relative to a control, were elevated liver iron, decreased blubber thickness, and the decreased ratio of the sum of PCB congeners/sum of PBDE congeners. The results of this study suggest that mucormycosis may pose an inordinately high risk to harbor porpoises (and potentially sympatric species in the Salish Sea such as southern resident killer whales) based on the

detected prevalence and the severity of lesions observed at necropsy. However, the risk may be greater on an individual basis compared to the overall population, and is likely related to other factors such as increased POP and heavy metal burdens.

KEYWORDS

harbor porpoise, fungi, killer whale, marine mammal, mucormycosis, One Health, qualitative risk assessment, Salish Sea

1 Introduction

Fungi of the order Mucorales are ubiquitous filamentous saprobionts and opportunistic organisms found in all climatic zones, containing approximately 50 genera and 300 species (Migaki and Jones, 1983; Petrikkos et al., 2012). These fungi are a One Health concern due to their ability to infect humans, other animals, plants, and persist in the environment in decaying organic matter such as leaves, soil, rotting wood and other vegetation, and dung (Migaki and Jones, 1983; American Society for Microbiology (ASM), 2019; Hassan and Voigt, 2019). Historically, mucormycosis has been called phycomycosis, and more recently zygomycosis, and is a disease caused by any species of the order Mucorales. Despite the pervasiveness of these fungi, the disease itself represents only 8-13% of all fungal infections in humans. Although Mucorales only represent a small portion of fungal infections, the mortality rate associated with them is striking (Roden et al., 2005) and increasing (Spellberg et al., 2005; Petrikkos et al., 2012). Infection in animals and humans is caused by inhalation of sporangiospores, direct inoculation into the skin, or ingestion, and is not considered zoonotic, nor does it transmit between individuals. In susceptible individuals the agent is aggressive and typically disseminates quickly throughout the body, invading blood vessel walls, resulting in thrombosis, infarcts (necrosis), and granulomatous inflammation.

Because mucormycosis is so aggressive, it is difficult to treat and often fatal (mortality >40%) (Roden et al., 2005). Though it occurs rarely in healthy individuals, mucormycosis is largely considered an opportunistic rather than primary pathogen in humans and animals. Infections may result from localized traumatic damage to the skin or generalized immune system suppression from chronic pre-existing disease or elevated contaminant levels (Ross, 2002; Desforges et al., 2016), such as humans with unregulated diabetes, HIV/AIDS, intravenous drug use, malnutrition, pregnancy, traumatic injuries and increased circulating levels of heavy metals such as iron or uptake of zinc, and/or coinfection with other infectious pathogens (Roden et al., 2005; Gomes et al., 2011; Petrikkos et al., 2012; Binder et al., 2014; Mouton et al., 2015; Wilson, 2015; Weiss and Carver, 2018). In humans, iron overload is a risk factor for mucormycosis (Petrikkos et al., 2012). A recent study in stranded false killer whales (*Pseudorca crassidens*) detected a strong link between opportunistic cutaneous fungal invaders and elevated levels of aluminum, selenium and zinc (Mouton et al., 2015). Although little is known about metal and trace element concentrations in marine mammals of the Pacific Northwest, a few studies have examined metals in harbor seals where regional differences in metal levels have been documented (Calambokidis et al., 1984; Calambokidis et al., 1991; Akmajian et al., 2014). Essential elements (e.g., zinc, copper, selenium) occur naturally in the environment and many have important biological functions, but excesses or deficiencies can have adverse health effects. Non-essential elements such as lead, cadmium and mercury can be toxic, depending on concentration and form, with evidence suggesting a possible link between long-term exposure to heavy metals such as mercury and infectious disease in harbor porpoises (Bennett et al., 2001; Wintle et al., 2011), immuno- and genotoxic effects in cetaceans (Kershaw and Hall, 2019), and more numerous lesions in dolphins with elevated cadmium and selenium levels (Monteiro et al., 2020). Underlying viral infections were also investigated considering herpesvirus was detected in harbor seals with mucormycosis and could be associated with the presence of the disease as well as a killer whale (*Orcinus orca*) under human care (Abdo et al., 2012b; Huggins et al., 2020).

In marine mammals, mucormycosis is reported less commonly than in humans, and is most frequently identified as those in human care, with a few isolated cases among wild, stranded individuals. It is unclear how marine mammals are exposed to Mucorales spp., but is suspected to be similar to humans with exposure to detritus containing the organism carried by winds or dispersal into the marine environment through flooding. The disease has been documented in delphinids, large whales, and pinnipeds (Kaplan et al., 1960; Best and McCully, 1979; Wünschmann et al., 1999; Robeck and Dalton, 2002; Naota et al., 2009; Morris et al., 2010; Barnett et al., 2011; Abdo et al., 2012a; Abdo et al., 2012b; Sosa et al., 2013;

Isidoro-Ayza et al., 2014; Huckabone et al., 2015; Nakagun et al., 2018; Huggins et al., 2020).

Over the past 20 years, increasing numbers of harbor porpoise (*Phocoena phocoena*) have been documented in the northern Salish Sea (Washington State, USA), with expansion southward into Puget Sound proper (Evenson et al., 2016; Jefferson et al., 2016). Though significantly impacted by negative environmental factors such as pollution and entanglement in fishing gear, harbor porpoises are not currently considered a threatened or endangered species, and are relatively abundant throughout the Salish Sea and along the outer Pacific Northwest coast (Evenson et al., 2016; Jefferson et al., 2016; Carretta et al., 2017; Morin et al., 2021). Harbor porpoises are a sentinel species in other regions of the world such as the Baltic Sea (ASCOBANS, 2010) and Canada (COSEWIC, 2016). Investigations into the disease and health conditions of harbor porpoises are revealing diseases that are unusual, increasing, or not previously recorded in the region (Huggins et al., 2015). For example, mucormycosis cases were noted for the first time in 2012 in a harbor porpoise (Huggins et al., 2020). Between 2012 and 2019, 21 cases of mucormycosis were detected in marine mammals, 19 confirmed and 2 suspect; in 12 adults, 6 subadults, and 3 pups, including 15 harbor porpoises, 5 harbor seals (*Phoca vitulina*) and an endangered southern resident killer whale (SRKW), the latter following deployment of a LIMPET satellite tag into the dorsal fin (Department of Fisheries and Oceans Canada (DFO), 2016; National Marine Fisheries Service, 2016a; National Oceanic and Atmospheric Administration, 2016b; Huggins et al., 2020). Both sexes and all seasons were represented (Huggins et al., 2020). Cases increased over time and peaked in 2016 ($n = 6$), the year that coincided with the first documentation of the fungus in species other than harbor porpoise. Most of the cases in this study occurred in inland waters of Washington State and British Columbia, Canada (Figure 1).

Harbor porpoises in the Salish Sea are ideal indicator species for monitoring marine ecosystem health, including emerging pathogens such as Mucorales (Moore, 2008; Bossart, 2011). This is especially relevant for the endangered SRKWs within the Salish Sea, whose small population size, and thus vulnerability to disease, was identified as one of the important concerns for this population's survival (NMFS, 2008; NMFS West Coast Region, 2016). SRKWs are a Vital Signs indicator for the Puget Sound Partnership to monitor the health of the Puget Sound ecosystem (Kershner et al., 2011). Given the prolonged timeframe, to acquire a sufficient sample size of tissues from killer whales for disease or contaminant analysis, along with the logistical challenges of studying their health, a more practical, efficient method is to monitor alternative surrogate species, such as harbor porpoises, that are easier to monitor and sample (Knap et al., 2002; Rabinowitz et al., 2005). Porpoises are sympatric with SRKWs, display inshore distribution, urban ecosystem residency, proximity to human populated areas, and greater abundance than the latter (Calambokidis et al., 2015; COSEWIC, 2016). Harbor porpoises and SRKWs eat high on the marine food web and display relatively strong regional site fidelity (Carretta et al., 2017). Thus, harbor porpoises are ideally suited to study for emerging pathogens that could affect SRKWs.

The relationship between environmental and demographic variables in harbor porpoise and the occurrence of mucormycosis is currently not well understood. Understanding how these variables influence development of this fungal disease in porpoise is key to identifying ways to attenuate the number of new cases that develop each year and to help mitigate exposure or development of the disease in endangered species that inhabit the same waters as porpoise, such as SRKWs. Our preliminary data identify an unusually large number of mucormycosis cases in harbor porpoises compared to other species (Huggins et al., 2020). Among the porpoise cases, animals have been emaciated, lactating ($n = 2$), affected with concurrent disease or heavy parasitism, pregnant ($n = 1$), or free of any obvious underlying disease.

Based on the varied presentation of cases, the contributors to mucormycosis appear to be multifactorial and likely similar to those in humans; therefore, we set out to investigate whether any of the following potential risk factors are associated with infection caused by mucormycosis: elevated pollutants and trace metals (e.g., iron), co-infection with other pathogens (e.g., virus, bacteria), emaciation, or pregnancy and lactation, as well as environmental variables such as heavy precipitation in the Salish Sea region (Nnadi and Carter, 2021). Environmental disruptions due to climate change, such as more severe flooding and other natural disasters, can readily aerosolize and disperse fungal spores, which are small, throughout the environment. This is particularly true when the spores encounter favorable climatic conditions such as lower rainfall and higher temperatures (Richardson, 2009).

Qualitative risk assessments serve as initial steps in assessing the risk of a specific hazard such as acquiring a pathogen (Jakob-Hoff et al., 2014). Risk assessments help contribute to risk management by breaking down threats into categories that are identifiable, and define the impact of each risk or risk factor (Graves, 2000). Quantitative risk assessments provide more objective information and accurate data than qualitative risk assessments because the former are based on data that are measurable and often more realistic than the latter and are, thus, often preferred. However, quantitative risk assessments are often impractical or impossible to perform due to a lack of measurable data on which to obtain a valid, numerical health risk assessment (Graves, 2000; Leighton, 2002). Because there is a lack of accurate numerical information on mucormycosis in marine mammals, much less harbor porpoise, to perform a quantitative assessment, a qualitative risk assessment can be performed instead. It is easily performed, quick to implement, and practical due to the lack of statistical/numerical dependence (Leighton, 2002). Thus, our aims for this project were two-fold:

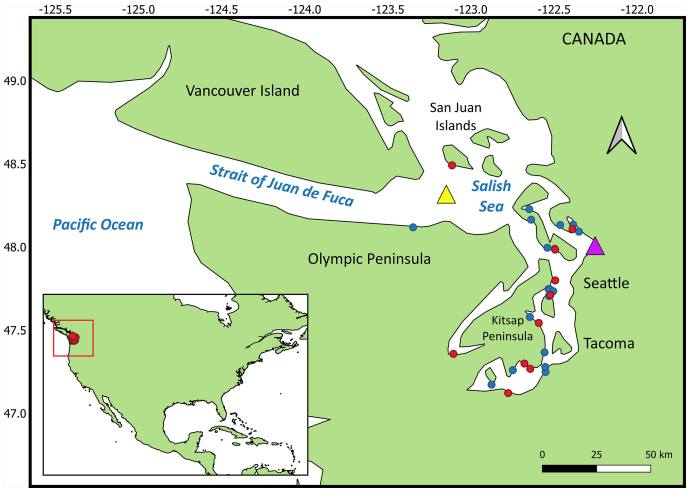

FIGURE 1
Mucormycosis cases (red dots) and controls (blue dots) in harbor porpoise. The yellow triangle is the location of the buoy that recorded mean yearly sea surface temperature. The purple triangle is the location where mean yearly precipitation was recorded.

to evaluate risk factors that might be associated with mucormycosis in harbor porpoise, with the intent of improving our ability to identify marine mammals at risk for the disease; and second, perform a qualitative risk assessment for mucormycosis in harbor porpoises within the Salish Sea.

2 Methods

2.1 Risk factor analysis

2.1.1 Study population and case/control selection

Study subjects were identified from the records of the National Oceanic and Atmospheric Administration (NOAA Fisheries), West Coast Region marine mammal stranding network, over the study period (2012-2019). Marine mammal stranding response groups collect dead beach cast animals, conduct examinations, and in many cases, complete necropsies and tissue sampling. The focus of this study was on harbor porpoise given the relatively high number of annual strandings and the overrepresentation of mucormycosis in this species (Huggins et al., 2020). Records of stranded harbor porpoises within the Salish Sea were thus extracted, from which cases and controls were selected. A confirmed case was defined as a harbor porpoise that stranded within the Salish Sea, presented with suspected findings at necropsy (e.g., marked swollen lymph nodes and/or presence of masses within organs), the pathogen detected at histopathological examination, with confirmation by culture and/or molecular sequencing. Controls were defined as a harbor porpoise that stranded within inland Washington State waters, and was negative for any histopathological or diagnostic findings as described for cases. In addition, the cause of death in controls must have been unrelated to fungal disease to avoid selection bias. Analyses were completed for potential risk factors using samples collected from fresh, dead-stranded harbor porpoise mucormycosis cases and controls within the Salish Sea.

Analyses of the following potential risk factors for mucormycosis were completed in cases of mucormycosis (n = 10) and controls (n = 20), (n = 30 total for study): persistent organic pollutants (POPs), and trace metals concentrations, and pathogen (viral and bacterial) screening. For all study samples, one-gram sections of blubber were collected from fresh stranded carcasses and frozen until analyzed for POPs and lipid content at NOAA's Northwest Fisheries Science Center (Seattle, WA). The POPs included the sums of chlordanes (CHLD), dichloro-diphenyl-trichloroethane (DDT), hexachlorocyclohexanes (HCH), 46 polychlorinated biphenyl (PCB) congeners (as 40 chromatographic peaks), a subset of the PCB congeners (105, 118, 156) for which toxic equivalency factors were available to us, and 15 polybrominated diphenyl ether congeners (PBDEs). In addition, the following POP ratios were evaluated as potential risk factors: the sum of the 46 PCB congeners as a ratio to each of the sums of CHLDs, DDTs, HCHs, and PBDEs, respectively. Blocks of frozen liver samples from all cases and controls were submitted to ALS Environmental (Kelso, WA) and the California Animal Health and Food Safety Laboratory at University of California (Davis, CA) for the following trace metals/elements that serve important roles in the survival and the pathogenesis of invasive fungal disease (Gerwien et al., 2018):

aluminum (Al), arsenic (As), copper (Cu), cadmium (Cd), iron (Fe), nickel (Ni), silver (Ag), lead (Pb), mercury (Hg), selenium (Se), and zinc (Zn).

To determine the presence of co-morbidity with infectious/noninfectious diseases, detailed necropsies were conducted on the fresh harbor porpoise carcasses following standard techniques (Geraci and Lounsbury, 2005). Histological examination was completed on all cases and controls at Northwest Zoopath (Monroe, WA), and Animal Health Center (Abbotsford, BC). Histochemical (Grocott methenamine silver, acid fast, and Gram) and immunohistochemical stains were used as needed and previously described in harbor porpoise mucormycosis cases (Huggins et al., 2020). Frozen tissues were submitted to the One Heath Institute Laboratory at the University of California (Davis, CA) for screening for morbilli- and herpesviruses by reverse transcriptase and standard polymerase chain reaction (PCR), respectively, based on established methods (Van Devanter et al., 1996; Tong et al., 2008). PCR for Brucella was conducted by the Veterinary Diagnostic Laboratory at the University of Illinois (Urbana, IL).

2.1.2 Specification of levels of potential risk factors

Descriptive data with no numeric value or order (nominal risk factor) included sex; age class (subadult or adult); and season of stranding - winter (December-February), spring (March-May), summer (June-August), and autumn (September-November). The binary data were recorded as yes versus no, unless otherwise indicated (e.g., sex) and included comorbid lesions present; and age class. Continuous variables included lateral blubber depth at maximum girth (cm); the POPs as listed under section 2.2; location, in decimal degrees latitude, of the stranding north or south relative to the approximate lateral midpoint of the Salish Sea (latitude 48°N; roughly at Everett, WA); and environmentally acquired metals that are deemed of importance to homeostatic regulation in pathogenic fungi, for which a sufficiently large body of literature exists, such as iron, zinc, and copper (Gerwien et al., 2018). Decimal degrees relative to latitude 48°N was selected as a variable because past evidence along the outer Washington coast suggested ratios of select contaminants, such as PCB and DDT in harbor porpoises, are associated with latitude (location), indicating this species' movements may be restricted in some areas, helping to identify the geographic region from which the porpoise originated (Calambokidis and Barlow, 1991), as has been done in SRKWs (Krahn et al., 2007). Mean yearly sea surface temperature data for the Salish Sea were obtained for a buoy moored in the eastern Strait of Juan de Fuca (48.332° N, -123.179° W) (yellow triangle, Figure 1) for 2012-2019 and were reported as °C (National Oceanic and Atmospheric Administration, 2020a). Mean annual precipitation (cm), for the year preceding each mucormycosis case was reported, was obtained from a precipitation monitoring station approximately mid-Salish Sea, at Everett, Washington (purple triangle, Figure 1) (National Oceanic and Atmospheric Administration, 2020b). The outcome (dependent) variable for the condition regression model was case/control status.

2.1.3 Molecular identification

Molecular genotyping was performed in eight of the 10 cases and four of the control porpoises (retrospectively) at NOAA Fisheries' Northwest Fisheries Science Center (Seattle, WA) to confirm (or rule out) the fungal family and/or species, and has been recently described in more detailed elsewhere (Huggins et al., 2020). Not all cases and controls were molecularly tested due to confirmation by other methods (e.g., histopathology). Targeted tissue samples from suspected case animals, that included those stored in RNAlater®, in paraffin blocks, or frozen in Whirl-Pak bags, were analyzed using polymerase chain reaction and sequencing.

2.1.4 Statistical analyses

To determine the presence and strength of association of the independent potential risk factors with confirmed mucormycosis, a matched case-control study was implemented using conditional logistic regression with two controls matched by age class to each case (Pearce, 2016). Matching was used to reduce confounding by the independent variables and to gain greater statistical efficiency and precision (Rose and van der Laan, 2009; Pearce, 2016). Conditional univariate logistic regression was performed to identify those independent variables associated with the dependent outcome variable (disease status) to determine if the variable would be included in the initial full model. Risk factors with $P < 0.20$ on univariate analysis were eligible for inclusion as were any potential 2-way interaction terms that were deemed biologically plausible (e.g., interaction between sex and levels of total CHLD, DDT, HCH, PBDE, and PCB, as well as degrees latitude from mid-Salish Sea and POPs). A threshold of $P < 0.20$ was chosen to prevent exclusion of a potentially marginally significant risk factor that may only become significant when in the presence of other risk factors during the multivariable analysis (Dohoo et al., 2003). Collinearity between related variables, such as POPs and POPs with degrees latitude of stranding relative to mid-Salish Sea, was examined *via* the Pearson correlation coefficient (r). When variables were strongly correlated (i.e., $r > 0.70$), the one with the greatest odds ratio (OR) on univariate analysis was kept for modeling purposes and the other(s) were excluded.

We used a manual, stepwise approach to model building. Independent variables at $P < 0.20$ were kept, and the effect of adding or subtracting each variable was examined for evidence of confounding, represented by a change in other covariate coefficients by more than 20%. Akaike information criterion (AIC) values were used to select the final model out of all candidate models (Akaike, 1974). The Hosmer-Lemeshow test

assessed final model goodness-of-fit. Results of conditional logistic regression analysis were reported as ORs and 95% CIs. The OR represents the relative change in odds for mucormycosis associated with the presence of a given factor(s) or, for continuous variables, for each (single) unit change in that factor (e.g., precipitation). Odds ratios are used to quantify the magnitude of association between a potential risk factor and the outcome (disease). Therefore, when an OR = 1, there is no association; an OR > 1 indicates the risk factor is associated with an increased in odds of the outcome and the reverse for OR < 1.

A secondary regression analysis, using multiple linear regression (MLR), was used to evaluate the association of levels of various contaminants with independent risk factors (sex, length, age class, blubber thickness, mucormycosis status, degrees latitude distance relative to 48°N, season, and lipid percent). The dependent outcome variables were lipid-normalized sums of concentrations for each POP class (PBDE, CHLD, DDT, HCH, PCB). We explored a number of contaminant ratios, including the ratios of summed PCBs to those of PBDE, CHLD, DDT, and HCH. The resulting beta coefficients were standardized to evaluate which of the independent variables have a greater effect on the dependent variable since the independent variables are given in different units of measurement. The beta coefficients are essentially considered a general measure of effect size, or magnitude of the effect of one variable on another.

2.2 Qualitative risk assessment

Due to limited data on mucormycosis in harbor porpoise, a qualitative, versus quantitative, risk assessment approach was selected to determine the probability of risk to harbor porpoises of acquiring mucormycosis. We utilized a qualitative risk assessment based on the World Organisation for Animal Health (OIE) and Food and Agriculture Organization-World Health Organisation (FAO-WHO) (Food and Agriculture Organization of the United Nations/World Health Organization, 2011) assessment frameworks, incorporating available data from published and unpublished sources. This type of assessment consists of identifying the probability of a risk event (i.e., transmission of the disease agent), and the impact the risk will have if said event does occur (i.e., recipient of disease agent becomes infected). A framework for this approach has already been developed and used by multiple agencies for assessing animal diseases (Palmer et al., 2005; Heller et al., 2010; Food and Agriculture Organization of the United Nations/World Health Organization, 2011; Sharma et al., 2018). The two principal components of a qualitative risk assessment framework (Figure 2) include: (1) hazard identification (blue box) and (2) risk assessment (green box). The latter further consists of hazard characterization (pink boxes), exposure assessment (gold boxes), and risk characterization (purple boxes). All risks have both probability and impact. Once the hazard is identified, in this case mucormycosis in harbor porpoise, the overall risk can be assessed as a function of: a) the probability that a porpoise will be exposed to the fungi (exposure assessment); and b) the magnitude of the consequences of such a hazard (hazard characterization). The resulting probabilities (exposure and consequence) are then combined to give an overall qualitative estimation of risk using a matrix approach (risk characterization).

Because relatively little information on fungal organisms in marine mammals exists, compared to terrestrial animal species or humans, data used to assess the risk were collected from published and unpublished sources. Information on health effects from, and exposure levels to, mucormycosis were extrapolated from the marine mammal literature where available, and otherwise from terrestrial species such as domestic animals and humans. Key words including 'marine mammals', 'porpoise', 'dolphins', 'cetacean', 'seal', 'sea lion', 'pinniped', 'fungi', 'mucormycosis', 'zygomycosis', 'terrestrial', 'transmission', and others were searched using PubMed and Web of Science in November 2019. Each publication was screened for its relevance to transmission, lesions, mortality, and clinical manifestation of mucormycosis. Health effects and exposure levels from other marine mammals and humans were extracted from the publications for guidance in developing the current risk assessment framework.

The categories of disease severity and probabilities of exposure were qualitatively assessed using a descriptive scale adapted from Zepeda-Sein (2002), according to four different levels of risk. The qualitative levels were determined subjectively according to the following definitions: negligible (the probability of occurrence of the event is sufficiently low to be ignored, or to be considered only in exceptional circumstances; low (the occurrence of an event is a possibility in a minority of cases); moderate (the occurrence of the event is a possibility in the majority of cases); and high (the occurrence of the event is probable), and were assigned disease severity scores of one, two, three, and four, respectively. The categories of exposure to Mucorales were expressed as prevalences (%) on the following scale: negligible; low; moderate; and high, likewise assigned to one through four, respectively. Lastly, the risk categories for disease severity and exposure risk were subsequently combined with each other to get a total combined qualitative estimation of risk of mucormycosis specific to harbor porpoise. The assignment of each total estimated risk value was also subjective and completed by the first author (SAN) only. The assignment of scores were derived from "clinical" manifestations of mucormycosis, of which the closest we could obtain were gross and histopathologic lesions and information from the few published clinical cases in marine mammals under human care. Baseline levels of iron and other heavy metals are unknown in this species, so could not be used as part of the hazard

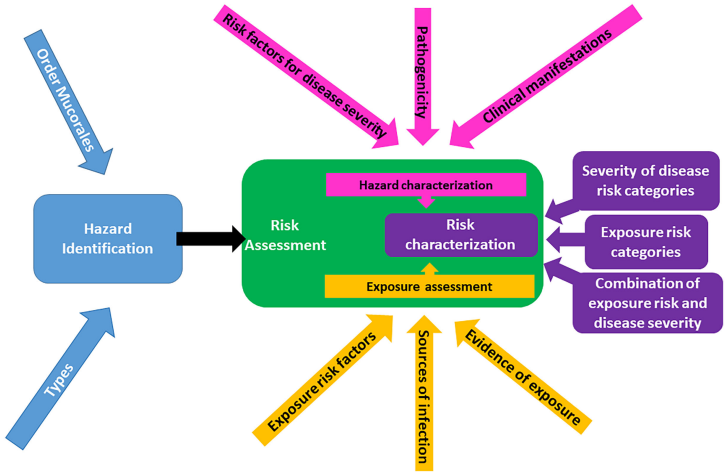

FIGURE 2
Structural framework for a qualitative risk assessment of mucormycosis in harbor porpoises, demonstrating the three major steps of the assessment: hazard identification (blue), risk assessment (green), and risk characterization (purple). Adapted from Food and Agriculture Organization of the United Nations/World Health Organization (2011) and Sharma et al. (2018).

characterization. Hence, we ran the separate potential risk factor evaluation (regression) to identify possible factors to focus on in the future that could be used in future assessments.

3 Results

Cases (and controls) were represented by five males, five females (10 males, 10 females) and eight adults, two subadults (14 adults, six subadults), respectively (Supplementary Table 1). Mucormycosis was determined to be the primary cause of mortality in eight of the ten cases in which it was detected, and a secondary contributory to mortality in two of the ten. Many of these animals had other illnesses and/or poor body condition. Three adult females were lactating, but not pregnant, whereas another was pregnant, but not lactating. Multisystemic parasitism was common, and one porpoise was co-infected with encephalitis due to *Toxoplasma gondii*. One infected harbor porpoise was collected after researchers witnessed it being attacked and partially consumed by transient killer whales; the attack itself was the cause of mortality, but we were unable to determine the extent of the fungal infection from the remains.

3.1 Risk factor analysis

3.1.1 Matched case-control study

Assessment for collinearity left the following potential risk factors available for univariate analyses: sex, iron (Fe), copper (Cu), zinc (Zn), season, mean annual precipitation the year preceding the stranding, mean annual sea surface temperature the year of the stranding, year of stranding, body condition, maximum blubber thickness at necropsy (maxblub), sum of DDTs (sumDDT), sum of the PCB congeners 105, 118, 156 (sumCB105118156), the ratio of sum PCB/sum HCH (ratioPCB/HCH), the ratio of the sum PCB/sum PBDE (ratioPCBPBDE), presence of co-morbidities, the degrees of latitude north or south relative to mid-Salish Sea (latitude 48°N), straight length, and lipid percent in the blubber. Following univariate analyses, the full conditional logistic model contained the independent variables Fe, Cu, Zn, blubthick, and ratioPCBPBDE (Table 1). No interaction terms were deemed influential during model development. The best model fit based on AIC was the model with the variables Fe, blubber thickness, and the ratio of the sum of PCB/sum PBDE.

The ORs serve as an approximation of "relative risk". For example, an increase in Fe by a unit increases by two percentage points the probability that a harbor porpoise had mucormycosis (Table 2). Though a small percentage increase in probability, the small sample size and other unknown covariates may dampen the strength of the association between Fe levels and mucormycosis. The variable blubber thickness was marginally significant and may represent a biological surrogate for depletion of stored contaminants from the blubber as it is utilized that are released into the blood stream, along with the possibility of metabolism of these contaminants into compounds with higher toxicity, such as hyrdroxy-PCBs and -PBDEs.

3.1.2 Evaluation of contaminants (multiple linear regression)

Ten models of MLR were created that evaluated the relationship between the various types of POPs and the

TABLE 1 Evaluation of conditional logistic regression models of mucormycosis case status (case or control).

Model predictors for mucormycosis status	Model	Residual d.f.	Log-likelihood	AIC	LR	LR d.f.	P-value
Fe + Cu + Zn + blubthick + ratio PCBPBDE	Full	5	-6.170	24.340	–	–	–
Fe + Zn + blubthick + ratio PCBPBDE	A	4	-6.499	22.997	0.66	1	0.417
Fe + Cu + blubthick + ratio PCBPBDE	B	4	-6.231	22.462	0.12	1	0.727
Fe + blubthick + ratio PCBPBDE	C	3	-6.566	21.133	0.79	2	0.672

Fe, iron; Cu, copper; Zn, zinc; blubthick, lateral thickness of blubber at maximum girth; ratioPCBPBDE, ratio of sum PCB/sum PBDE.
Reduced models were compared to the full model using likelihood ratio (LR) tests (LR; χ^2-squared distributed) and Akaike Information Criterion (AIC) after sequentially dropping covariates and assessing significance (P <0.05). Final model in bold; d.f., degrees of freedom.

independent variables: sex, length, age class, season, blubber thickness, mucormycosis status, year of stranding, latitude north or south relative to mid-Salish Sea (latitude 48°N), and lipid percent of the blubber sample (Supplementary Table 2). The association between POP type and the independent variables was significant multiple times for sex, age class, and blubber thickness. Although we explored a number of contaminant sums and ratios the only one that showed any significant association with mucormycosis was for the sum of the PCB congeners 105, 118, 156 (P < 0.001). When the POP was a ratio of two contaminants, sex was still often significant, but the latitude relative to mid-Salish Sea was significant for three of the four ratios.

3.2 Qualitative risk assessment

The results from the assessment follow the flow diagram depicted in Figure 2 with each section below referring to the corresponding section in the diagram.

3.2.1 Hazard identification

The resulting risk analysis framework (Figure 2) was based only on cases of mucormycosis in harbor porpoise, due to the paucity of data in other affected species, such as harbor seals and killer whales. In the present assessment, the hazard is identified as mucormycosis (Figure 2, blue boxes).

3.2.1.1 Types

Fungal speciation was determined in five cases and has been described previously (Huggins et al., 2020). The results of PCR sequencing of frozen tissues detected three cases of Rhizomucor pusillus and one of Lichtheimia corymbifera. Culture of tissues from one porpoise resulted in scant growth of the species Cunninghamella bertholletiae, which was identified by phenotypic characterization, and DNA sequencing.

3.2.1.2 Transmission modes, sources, and seasonality

Although the major mode of transmission in many fungi is aerosolization of their sporangiophores, the mode(s) of transmission and seasonality for harbor porpoises remains undetermined. The modes were assumed to include inhalation of airborne spores, ingestion, or disruption of the cutaneous skin or mucosal barrier.

3.2.2 Risk assessment

The risk assessment (Figure 2 – green box) component of the assessment framework was composed of three parts: 1) hazard characterization; 2) exposure assessment; and 3) risk characterization, the results of which follow this order.

3.2.2.1 Hazard characterization

Characterizing the hazard (Figure 2 – pink boxes) resulted a summary of the known effects of mucormycosis in marine mammals based on published reports, to determine the pathogenicity and risk factors for mucormycosis disease severity. The most common gross lesions noted in the present porpoise cases were associated with respiratory disease and associated lymph nodes, and if disseminated, included masses in other organs such as brain, kidney, and spleen among other organs. Histologically, vascular disease was noted in the present harbor porpoise cases. These lesions were similar to those noted

TABLE 2 Maximum-likelihood estimates and odds ratio (OR) from fitting final conditional logistic regression model for a case-control study, matched on age class, to evaluate variation in mucormycosis status (case or control) associated with iron (Fe), blubber thickness (cm) at necropsy (blubthick), and the ratio of the sum PCB/sum PBDE (ratioPCBPBDE) in harbor porpoise (*Phocoena phocoena*) in the Salish Sea.

Variable	Estimate	OR	SE	Z value	*P*-value
Fe	0.017	1.020	0.009	2.07	**0.038**
blubthick	-3.792	0.023	0.045	-1.89	**0.052**
ratioPCBPBDE	-0.900	0.406	0.225	-1.62	0.104

Fe, iron; blubthick, lateral thickness of blubber at maximum girth; ratioPCBPBDE, ratio of sum PCB/sum PBDE.
Significant predictor coefficients (P < 0.05) are shown in boldface type. SE, standard error.

in the marine mammal literature. However, there were relatively few reports of mucormycosis in marine mammals, many of which occurred in stranded animals such as the present cases, which are often affected with multiple contributors to mortality. This resulted in potentially confounding results of disease interpretation and uncertainties as to the contribution of Mucorales species to specific lesions.

3.2.2.1.1 Clinical manifestations

Clinical signs of infection vary depending on affected organs and the severity of infection. Given the inability to clinically observe free-ranging harbor porpoises affected with mucormycosis antemortem, we report lesions noted on necropsy and histological examinations. Of the ten mucormycosis cases in our study, three were in poor and 5 in fair body condition, respectively (Supplementary Table 1). In addition, the cases presented with fungal lesions in the brain (8/10), lymph nodes of various locations (5/10), and the lungs (7/10), and if observed alive, would have likely manifested clinical signs related to these organ pathologies. All 30 study animals tested negative for *Brucella* spp. and herpes-/morbilliviruses. The two most common co-morbidities detected in the study animals (n = 10 cases/20 controls) were parasitism, most frequently liver flukes and/or lungworms, as a contributory cause of death (n = 5/13) and infectious disease due to bacteria or viruses other than *Brucella* or herpes-/morbilliviruses (n = 1/7) (Supplementary Table 1). Protozoal organisms were contributory causes of death in one case (morphologic features most consistent with *Toxoplasma gondii*) and one control (unspecified protozoa).

Microscopically, lesions may vary depending on location and chronicity. Acute lesions are associated with necrosis of small vessel walls, thrombosis, and fungal elements within the thrombi, vessel walls and adjacent tissue, with associated hemorrhage, tissue necrosis and neutrophilic or eosinophilic inflammation. Inflammation becomes pyogranulomatous with lesion chronicity (see Isidoro-Ayza et al., 2014; Huggins et al., 2020). In the case that was co-infected with suspected *T. gondii*, at least three separate infectious disease processes were present, including pulmonary nematodiasis, a mycotic abscess in the brain, and meningoencephalitis associated with the protozoa. Another case presented with at least two separate infectious processes. Most of the pulmonary lesions were attributed to nematodiasis, but a large pulmonary abscess and the intraabdominal abscess were due to zygomycosis. Freeze artifact was substantial in this specimen and, in some areas, impeded microscopic interpretation. Though it was difficult to definitively determine the porpoise's nutritional status due to freeze artifact, the animal may have been in suboptimal nutritional status likely due to the underlying infectious processes. Many of the microscopic lesions in the cases confirmed mucormycosis and the grossly observed parasitism.

3.2.2.1.2 Risk factors for disease severity

In marine mammals, risk factors for mucormycosis may include underlying illness such as co-infection with viruses, malnutrition, elevated chemical contaminant loads, pregnancy, traumatic injuries, and excess iron. In harbor porpoises, detailed information is lacking on current POP loads and elemental metal accumulation, as well as the relation of these factors to the occurrence of mucormycosis in this species. Immunosuppressive or tissue damaging viral infections such as herpes- and morbillivirus can predispose a marine mammal to other pathogens such as fungi (Barrett et al., 1993; Arbelo et al., 2010; Van Bressem et al., 2014). Neither of these viruses was detected in the harbor porpoise cases of the present study. Given that normal ranges of metal and trace elements are unknown in most marine mammal species, including those of the Pacific Northwest, regional differences have been noted in a few studies in harbor seals (Calambokidis et al., 1984; Calambokidis et al., 1991; Akmajian et al., 2014), and may be the case with harbor porpoises.

3.2.2.2 Exposure assessment

This portion of the qualitative risk assessment (Figure 2 – gold boxes) evaluated the likelihood that the susceptible porpoise (s) would come into contact with the hazard (e.g., Mucorales fungi) that could potentially facilitate transmission; in other words, estimate the likelihood of susceptible animals being exposed to the fungi. The following sections list the relevant biological, ecological and geographical factors and the associated assumptions.

3.2.2.2.1 Exposure risk factors

None of the published case reports of mucormycosis included in this assessment directly studied risk factors for exposure to Mucorales in harbor porpoise. Therefore, we extrapolated from risk factors identified in other marine mammal species, other porpoise infectious diseases, and humans, and assumed they likely represented risk factors in harbor porpoise. Without numerical values to quantify exposure risk, the results of exposure risk are presented in terms of qualitative values. For example, the general likelihood of exposure for Mucorales as with most fungal agents was considered moderate to high due to the ubiquitous presence of these pathogens in the environment. Fungi in general are not considered to be transmitted between individuals, so close physical interaction with other marine mammal species in the Salish Sea would not be a risk factor for exposure. Chronic exposure to heavy metals such as mercury, selenium, and zinc may predispose to immunosuppression and thus be associated with increased susceptibility to infectious disease, so the risk may be considered at least moderate. The POP levels measured in this study's porpoises are not unexpected for the Salish Sea at the trophic level that porpoises feed. However, depending on their

site fidelity and prey migratory patterns, porpoises may be spending more time or feeding on prey in more urbanized areas in the southern part of Puget Sound. Therefore, these individuals will be exposed to higher levels of POPs, on average compared to those spending more time in northern Puget Sound. Exposure may only require a single respiration or ingestion of the organism in an immunosuppressed or otherwise stressed animal; therefore, mitigating exposure will be very challenging.

3.2.2.2.2 Sources of exposure

Routes of exposure for Mucorales in harbor porpoise and other marine mammals is not completely known, but the fungi could be introduced into the marine ecosystem from terrestrial sources (Richardson, 2009). With the predominant mode of acquisition of these fungal infections presumed to be inhalation, this route was assumed to be the case for the present cases. However, we could not rule out the possibility the fungi alternatively entered through trauma to the skin or mucosal barrier from injuries such as interspecific interactions or predatory trauma. Although it is also possible a marine mammal could become infected by ingestion of prey containing sporangiospores, ingestion is more likely to occur from direct incidental swallowing of marine water containing sporangiospores during acquisition or ingestion of prey, and could have occurred in our case animals.

A monthly survey of ~80 sites throughout Puget Sound over a seven-month period (April through October) of pelagic seawater at a six-meter depth detected only ten positives out of 547 samples using a dual detection quantitative PCR specifically for Rhizomucor and *Lichtheimia* (Salehi et al., 2016; LDR, unpublished data). Positive sites were distributed across the geographic extent of Puget Sound, and the majority occurred in May and August. Although these fungal genera may have low frequency in the pelagic water column of harbor porpoise habitat, surface water may have higher levels of airborne spores deposited as fallout. As previously hypothesized for porpoises, disturbance of soil or detritus by environmental or climatic events, may introduce spores into the marine environment by winds or water runoff from terrestrial sources (Huggins et al., 2020).

3.2.2.2.3 Evidence of exposure

Few studies have documented exposure or clinical disease in live, free-ranging marine mammals. *Cunninghamella bertholletiae* was cultured from the blowhole and stomach of a presumed healthy, wild bottlenose dolphin (*Tursiops truncatus*) sampled during a microbial culture survey in the waters off Charleston, South Carolina (USA) (Morris et al., 2010). Two cases of nasal mucormycosis were documented in harbor seal pups presenting to rehabilitation, with concurrent herpesvirus infection, in Vancouver, British Columbia (Canada) (Huggins et al., 2020).

3.2.2.3 Risk characterization

The next three subsections collectively formed the risk characterization (Figure 2 – purple boxes). The first two subsections, severity of exposure to the agent Mucorales and severity of mucormycosis, which were approximated, were combined to calculate an overall estimate of risk associated with mucormycosis, which essentially estimates the consequences of the hazard (i.e., mucormycosis) occurring in Salish Sea harbor porpoise.

3.2.2.3.1 Risk categories for exposure (exposure assessment)

Though the incidence of mucormycosis is increasing worldwide, its prevalence and incidence are difficult to estimate since it is not a reportable disease, is currently considered rare, and the risk varies widely in different populations of humans and animals (Gomes et al., 2011). We assumed similar low incidences as observed in humans. Seroprevalence data on exposure of marine mammals to Mucorales are poorly defined. For this study, we extracted prevalence data from those reported in other marine mammal species, and used those values in the assessment as part of characterizing the overall risk of mucormycosis. Values ranged from < 0.025% in California (Huckabone et al., 2015) and 0.23% in the Baltic Sea (Wünschmann et al., 1999) to 1.16% in the Salish Sea (Huggins et al., 2020). Thus, the categories of exposure to Mucorales were expressed as prevalences (%) on the following scale: negligible (0-0.1%); low (0.2-0.5%); moderate (0.6-0.9%); and high (> 0.9%) (Table 3). Based on these published prevalences, we assumed a moderate to high incidence, and thus prevalence, in harbor porpoises, and considered exposure risk to be moderate (prevalence 0.6-0.9%; Score 3) or high (prevalence > 0.9%; Score 4).

3.2.2.3.2 Risk categories for disease severity (hazard characterization)

Categorizing mucormycosis disease severity, based on the presence of gross and microscopic lesions, resulted in four categories: negligible (1), low (2), moderate (3), or high (4) (Table 3). Though clinical signs have not been documented in harbor porpoises, they have been observed in a few captive animals, so these categories assumed similar clinical signs would also be present in porpoises if ante-mortem observation was possible. Associated disease, without overt clinical disease, has been occasionally reported in Salish Sea harbor porpoises (Huggins et al., 2015). The severity of lesions observed in Salish Sea cases is such that it is assumed clinical signs were present, but not observed in the field; therefore, a risk category of (4) is assigned.

3.2.2.3.3 Overall risk characterization (combination of disease severity and risk of exposure scores)

Overall risk characterization scores were calculated by multiplying the disease risk scores by the exposure scores

TABLE 3 Risk categories for disease severity and exposure probability.

Risk category	Disease severity	Disease severity score	Prevalence (%)	Exposure (prevalence) score	Overall risk category
Negligible	No pathology or clinical signs	1	None 0-0.1	1	1-3
Low	Mild pathology (histological, no visible gross) but no clinical signs, and mild/no comorbidity	2	Mild 0.2-0.5	2	4-6
Moderate	Moderate pathology (visibly gross) with accompanying clinical signs and co-morbidity	3	Moderate 0.6-0.9	3	7-9
High	Severe pathology (visibly gross) and clinical signs and co-morbidity	4	High > 0.9	4	10-16

(from Table 3). The resulting overall risk scores could range from 1 to 16, with 16 being the worst possible outcome (Table 4 and Figure 3). Insufficient data are available to determine any finer scale risk scores. For Salish Sea harbor porpoise, a final risk score of 6-8 (Table 4), or low-moderate risk, was calculated because of the range of presentations of disease severity in this species. For SRKWs, calculated a final risk score of eight (Table 4), or moderate risk. However, based on information from mucormycosis in humans and other animals, if these two species have elevated iron levels and/or POP levels, and are immunocompromised by other disease processes, are likely at higher risk for developing moderate to severe disease from mucormycosis. There are data gaps that give this risk assessment a relatively high level of uncertainty such as poor knowledge of mucormycosis prevalence in Salish Sea cetaceans.

4 Discussion

We conducted a qualitative assessment of the risk of acquisition of mucormycosis in harbor porpoises between 2012-2019 in the Salish Sea. Results of the risk factor analysis indicated that elevated levels of iron in the tissues (and possibly blood), decreasing blubber thickness and decreasing ratio of total PCBs/PBDEs may be associated with mucormycosis and might serve as factors to include in future qualitative risk assessments for this disease. Based on 'clinical' (i.e., gross and histopathologic lesions) signs, and likely prevalences of exposure to the fungal agents, harbor porpoise, and thus killer whales, are currently at low to moderate risk for mucormycosis. The study provided an opportunity to obtain estimates of the potential overall risk of mucormycosis in harbor porpoises, and more importantly, to attribute qualitative risk rankings to the various components of the assessment such as disease severity and exposure risk. Our findings indicated that Salish Sea harbor porpoise may currently be at increased risk; however, there are a very limited amount of published data available so perceived risk may change in the future as more data are gathered on the ecology of this disease in harbor porpoise and their urban environment. However, seals and killer whales may be as susceptible to fungal disease as harbor porpoises and should not be ignored when assessing mucormycosis risk in the future. The study identifies data gaps that require attention and prioritization to increase the likelihood of future quantitative risk assessments.

Assessing the risks of mucormycosis in marine mammals is important from a health and conservation perspective. Given their coastal inland water home range and prey preferences, harbor porpoises in the Pacific Northwest are a good model to investigate pollutants and potential links to primary or opportunistic diseases, including mucormycosis. The emergence of Mucorales as a source of marine mammal mortality is of particular concern for nearby endangered cetacean populations, such as southern resident killer whales. We identified risk factors (Fe overload and potentially

TABLE 4 Overall risk characterization score for harbor porpoise in the Salish Sea and southern resident killer whales, for comparison.

	Hazard characterization		Exposure assessment		Risk characterization
Population	Presumed clinical manifestation (based on necropsy and histological findings)	Disease severity score *	Prevalence (%)	Exposure (prevalence) score #	Overall risk category ◊
Salish sea harbor porpoises	Moderate to severe - visibly gross signs of masses on internal organs such as lungs with presumed accompanying clinical signs (weight loss, respiratory difficulty), and comorbidity	3-4	Low (0.2-0.5%)	2	6-8
Southern resident killer whales	Moderate to severe - visibly gross signs of masses on internal organs such as lungs with presumed accompanying clinical signs (weight loss, respiratory difficulty), and comorbidity	4	Low (0.2-0.5%)	2	8

Disease severity and exposure scores are from Table 3. The symbols * and # indicate the values multiplied together to obtain the value denoted by ◊.

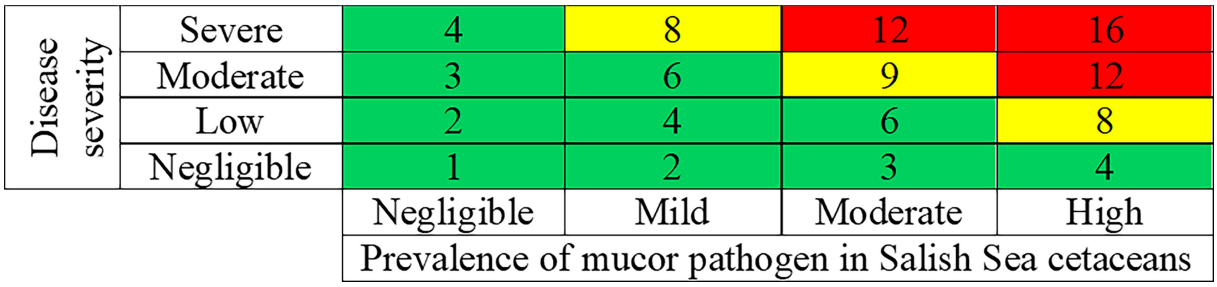

FIGURE 3
Risk matrix for combining the results of the disease severity scores (from Table 3) and the exposure scores (from Table 3), resulting in overall risk characterizations scores for mucormycosis in harbor porpoise. Green, yellow and red, respectively, represent negligible to low (combined together), moderate, and high risk.

contaminants) that should be more thoroughly investigated for future monitoring. Baseline porpoise and environmental prevalence data will be helpful to detect changes in the presence of this fungal organism in the Salish Sea ecosystem linked to anthropogenic or environmental forces. The presence of fungal species in marine waters does not appear to be high (~1,100 fungal species retrieved exclusively from the marine environment), with a relatively small percentage of described species associated with marine environments (Amend et al., 2019). Conspicuously little is known about the diversity and ecological functions of fungi in marine ecosystems, compared to their bacterial counterparts. If the pressure from fungi is not large, susceptibility to potentially pathogenic fungi in the host may be greater compared to bacteria because the host is not exposed to the former as often as the latter, and thus may be naïve to infections from fungi. Interactions among fungi and other marine biota likely have considerable implications extending beyond the individual host or local community (e.g., influencing the ocean geochemistry) (Amend et al., 2019).

Comprehensive health profiles demonstrate that the vulnerability of these animals to infectious diseases such as mucormycosis may be influenced by susceptibility to pathogens and the ability to cope with the infection (Hall et al., 2006; Egan and Gardiner, 2016; Hodges and Tomcej, 2016; Raverty et al., 2017). Pollutant loads in higher trophic level feeders such as harbor porpoise can reflect environmental burdens. Harbor porpoises and other regional marine mammals have accumulated POPs, such as PCBs and PBDEs (Calambokidis and Barlow, 1991; Ross et al., 2013). Though key viruses such as herpes- and morbillivirus were not detected in any of the study animals, two harbor seals were reported with nasal mucormycosis were co-infected with phocid herpesvirus (Huggins et al., 2020). A killer whale was co-infected with disseminated mucormycosis and a herpesvirus, distinct to phocine herpesvirus (Abdo et al., 2012b). Co-infection with bacterial microbes has been documented in animals affected with mucormycosis and may play a role in susceptibility to or disease severity (Wünschmann et al., 1999; Robeck and Dalton, 2002; Abdo et al., 2012a; Isidoro-Ayza et al., 2014; Huggins et al., 2020). In cases of disseminated mucormycosis lesions may also be seen in other organs such as the kidney (Sosa et al., 2013; Huggins et al., 2020), skeletal or cardiac muscle (Best and McCully, 1979; Wünschmann et al., 1999; Robeck and Dalton, 2002; Naota et al., 2009; Barnett et al., 2011; Abdo et al., 2012b; Huggins et al., 2020), central nervous system (brain) (Wünschmann et al., 1999; Robeck and Dalton, 2002; Barnett et al., 2014; Isidoro-Ayza et al., 2014; Huggins et al., 2020), and skin (Robeck and Dalton, 2002; Huggins et al., 2020).

The risk assessment identified several knowledge gaps which should be addressed and are similar to those identified for *Toxoplasma gondii* infection in Arctic beluga whales (*Delphinapterus leucas*) in Canada (Sharma et al., 2018). The gaps include major transmission routes of the various Mucorales species to marine mammals, risk factors that promote infection and establishment of the fungi in the animal, the role of terrestrial species, including humans, in contributing to the environmental distribution of the fungi, and variation in Mucorales genotypes within harbor porpoises compared to other marine mammal species including southern resident killer whales (Huggins et al., 2020). Mucorales spores are easily aerosolized and dispersed by the air and insects, and are taken up by the human or animal *via* inhalation, ingestion, or through disruption of the cutaneous skin or mucosal barrier, with the number of airborne spores dependent on the presence of climatic conditions that favor their dispersal and growth (Richardson, 2009). Mucormycosis agents are typically found in soil and dust, composting vegetation, on rotting fruit, during heavy excavation and construction, and in air conditioning filters. The increased temperatures present in composting organic substrates are selective for thermophilic species, such as some species of *Absidia*, *Mucor*, *Rhizopus*, and *Rhizomucor*. Other potential sources of Mucorales spores include dog skin,

bird feathers (e.g., poultry), rodent feces and natural disasters such as storms (Cabañes et al., 1996; Saidi et al., 2000; Lim, 2005; Stejskal et al., 2005; Richardson, 2009). Spatiotemporal investigations of the role of anthropogenic disturbance of soil and land use changes around the Salish Sea region are also needed to understand the ecology of these fungi. Monitoring mycobiomes along the coastline can help determine if clinically relevant fungal species are present and understand their community and environmental dynamics (Nilsson et al., 2019; Wunderlich, 2020). Though environmental sampling specific to fungi has not been conducted in the Puget Sound region, studies in other geographic regions revealed the following mucor genera: *Mucor*, *Rhizopus*, *Lichtheimia*, and *Cunninghamella* as being some of the most commonly isolated (Calvo et al., 1980; Mousavi, 2018). As currently observed and projected global temperatures are expected to rise, and shifts in precipitation patterns and extreme climatic events increase in frequency globally (IPCC, 2014) possible shifts in fungal risk for wildlife, including marine mammals may increase.

Mucormycosis has not previously been regarded as having a seasonal occurrence. For some molds in multiple United States geographical locations, atmospheric concentrations of spores were highest in the summer and autumn, while lowest in winter and spring (Shelton et al., 2002). In addition, results from other studies that specifically targeted mucormycosis suggest the organisms, and thus cases, follow the same general seasonal pattern (Talmi et al., 2002; Al-Ajam et al., 2006; Sivagnanam et al., 2017). Furthermore, a study of dog skin biota revealed a peak spore prevalence of Rhizopus and Mucor species in the summer and autumn, respectively (Cabañes et al., 1996). The predominant mode of acquisition of these fungal infections is presumed to be *via* the respiratory tract (Gomes et al., 2011). A few air sampling studies have detected Mucorales species, but little testing for Mucorales in marine ecosystem waters has been done (Raverty et al., 2017). Environmental air samples, obtained outdoors throughout an aquarium with cases of *Aspergillus fumigatus* in their collection of beluga whales were positive for *Mucor* spp. (Young et al., 1999). The ubiquitous nature and small size of Mucorales sporangiospores allows them to remain airborne for prolonged periods, which can increase the exposure risk. Though exposure to Mucorales sporangiospores is considered likely from the air during inhalation at the sea surface microlayer (Raverty et al., 2017), the fungi can also enter through trauma to the skin or mucosal barrier from injuries such as interspecific interactions, predatory trauma, or injections (Vainrub et al., 1988; Lim, 2005; Nakagun et al., 2018; Huggins et al., 2020). Ingestion of Mucorales fungi in the infected tissues of prey items can be another possible mode of exposure for carnivorous marine mammals. Gastritis associated with a nematode infection in the first stomach compartment of a harbor porpoise was suspected to be the origin of entry for systemic mucormycosis due to a *Rhizopus* spp. (Wünschmann et al., 1999). Cases of mucormycosis have been reported in immunosuppressed humans who ingested food or medicine contaminated with sporangiospores (Oliver et al., 1996).

Clinical signs attributed to mucormycosis have been reported in marine mammals (Robeck and Dalton, 2002; Naota et al., 2009; Barnett et al., 2011; Abdo et al., 2012a; Abdo et al., 2012b; Sosa et al., 2013; Barnett et al., 2014; Nakagun et al., 2018); however, clinical mucormycosis has not been reported in harbor porpoise. This is due to the fact that few harbor porpoise are kept in captivity or are rehabilitated as lesions associated with the fungi have been observed in dead stranded animals within the Salish Sea. It is likely that species of Mucorales would be detected in live, free-ranging Salish Sea harbor porpoise if their respiratory and gastrointestinal systems were sampled (Morris et al., 2010), and based on the presentation of live-stranded harbor seal pups with mucormycosis in a rehabilitation setting (Huggins et al., 2020). Grossly, mucormycosis lesions appear similar to other types of systemic fungal infections and consisted of variably sized often cavitating masses with a pulmonary predilection as noted in case reports of other affected marine mammal species (Kaplan et al., 1960; Best and McCully, 1979; Wünschmann et al., 1999; Barnett et al., 2011; Abdo et al., 2012a; Huggins et al., 2020). Clinical signs observed in marine mammals in managed care are nonspecific and include inappetence (Robeck and Dalton, 2002; Naota et al., 2009; Abdo et al., 2012a; Abdo et al., 2012b; Barnett et al., 2014), respiratory difficulties (Abdo et al., 2012a; Nakagun et al., 2018), renal failure (Sosa et al., 2013), dermatological (Robeck and Dalton, 2002; Barnett et al., 2011) and neurological abnormalities that include seizures, abnormal motor function, nystagmus, or dull mentation (Barnett et al., 2011; Nakagun et al., 2018). These neurological presentations may render harbor porpoise, and other marine mammals, susceptible to predation, stranding, or trauma from vessel or propeller strikes. Furthermore, inter- or intraspecific trauma to cutaneous or mucosal barriers to animals, such as commonly encountered in the daily lives of marine mammals, could serve as a portal for entry of Mucorales species, of which several have a propensity for cutaneous or subcutaneous tissues as well as from inhalation of the organism at the sea surface microlayer (Robeck and Dalton, 2002; Barnett et al., 2011; Gomes et al., 2011; Raverty et al., 2017; Huggins et al., 2020). Such dermal infections by mucormycosis can be a risk to marine mammal species in the Salish Sea, especially those under pressure from other sources of mortality and reproductive stressors such as the SRKW (Raverty et al., 2017).

Animals in captive aquatic facilities may be at increased risk because fungi are known to contaminate wet environments even after disinfection, the animals live in close contact with each other and share husbandry staff, food preparation areas, and ambient air (Young et al., 1999; Barnett et al., 2011). In humans, patients with hematological malignancies, immunosuppression, diabetes, and chronic corticosteroid therapy are at risk for disseminated infection caused by mucormycosis. However,

some Mucorales species can infect immunocompetent individuals, usually after traumatic injuries or injections are administered (Robeck and Dalton, 2002; Nakagun et al., 2018). Routes of exposure for Mucorales in harbor porpoise and other marine mammals is not completely known, but the fungi could be introduced into the marine ecosystem from terrestrial sources (Richardson, 2009).

The diagnosis of mucormycosis is difficult in free-ranging animals and is typically detected in moderate to severe infections identified at necropsy. Blow sampling is a potential way to detect pathogens in free-ranging cetaceans; however, smaller cetaceans such as porpoises might be more challenging to sample compared to larger species such as killer whales (Raverty et al., 2017). Blow sampling by drones offers a potential route to sample respiratory tracts for fungal organisms. This type of sampling helps with epidemiological studies to further investigate risk factors for mucormycosis. However, it is logistically and financially challenging to screen wild species for the purpose of obtaining high quality samples, so dead stranded animals will continue to be the prime source of tissues to study mucormycosis. Application of tools such as next-generation sequencing hold potential to provide more rapid and lower cost detection capabilities for more widespread environmental and tissue fungal screening around the Salish Sea (Zoll et al., 2016).

This matching case-control study results highlight the highly likely role that elevated liver Fe may contribute to or modulate mucormycosis in harbor porpoise as it does in human serum (Ibrahim et al., 2012; Kousser et al., 2019). In humans and animals that are considered healthy, limiting serum iron availability is a universal host defense mechanism against many microbes, but particularly fungi such as Mucorales because these fungi grow poorly in normal serum unless iron from exogenous sources are added (Artiss et al., 1982; Boelaert et al., 1993; Ibrahim et al., 2012). Though individually, Cu and Zn were significantly associated with mucormycosis status, this was not retained when other independent variables were considered in the modeling. The loss of significance of these metals may be due to confounding by other factors not considered in this study or from the small study sample size, despite matching two controls to one case to help with study efficiency. Given the strong association of mucormycosis with Fe (Tables 1, 2), it would be worthwhile to further investigate the effect of other metals on mucormycosis. The interpretation of iron levels relative to mucormycosis should be interpreted conservatively, as iron is sequestered following emaciation (a potentially immunosuppressive condition), and is also sequestered in various tissues during chronic infections, to limit iron metabolism by the infectious agent. Therefore, trace mineral levels and fungal infection may be associated through more than one process. Although increasing blubber thickness measured at maximum girth was significantly associated with the odds of being a mucormycosis case, this finding was likely confounded by other factors such as comorbidities, POP levels, or other undetermined factors. The ratio of PCB/PBDE as a risk factor for mucormycosis, although not significant in the final model, is more difficult to interpret because it is unknown whether a smaller or larger ratio of these POPs would be clinically or biological relevant to the animal.

In humans, mucormycosis is most commonly detected in individuals with compromised immune systems from organ transplants, chemotherapy, or chronic diseases such as unregulated diabetes (El-Herte et al., 2012; Petrikkos et al., 2012). While few of these conditions are ever observed in free-ranging cetaceans, potentially compromised immunity in stranded marine mammals has been most commonly related to elevated POP levels, presence of infectious or chronic diseases and starvation/emaciation/limited prey availability, so there may be some precedent for being predisposed to mucormycosis. The final conditional logistic regression model and the multiple linear regression models (MLR) suggest that contaminants may play a role in the pathogenesis of mucormycosis. However, caution must be exercised when interpreting the modeling results due to the small sample size and the fact that association does not equate to causation. There may be other confounding factors such as individual variability in contaminant loads, the animal's innate immunity, physiological make-up, and other biological determinants that can influence the association between contaminant load and mucormycosis status. The MLRs in which the ratio of two classes of POPs was the dependent output variable, and was significantly associated with distance away from the latitude (48°N) at mid-Salish Sea, suggest potential regional patterns (or gradients) of POPs present in harbor porpoises within the Sea (north versus south of the Sea's midpoint at 48° N). If this is the case, then latitude may simply be a proxy measure of the likelihood of exposure to POPs, particularly if these animals have a strong site fidelity to a relatively small range (Elliser et al., 2018; Elliser and Hall, 2021). A geographic association between POPs and latitude may also be influenced by urbanization and use patterns in the Salish Sea and should be further investigated (Ross et al., 2013), as mucormycosis has been associated with soil disturbances during anthropogenic land use alterations (Miller and Lodge, 2007; Richardson, 2009).

This qualitative risk assessment and risk factor analysis are a strong start to identifying the underlying environmental and anthropogenic perturbations that increase the risk of developing this aggressive disease in a marine sentinel species like harbor porpoise. Underlying contributors to infection are likely prevalent and geographically widespread in the marine ecosystem, suggesting locally resident endangered populations such as southern resident killer whales could also be at significant risk. Because Mucorales pose a health risk within animal, human, and environments, studying this group of pathogens benefits from a One Health approach in which contributions stem from all three health sectors.

5 Conclusion

Though Mucorales are considered relatively uncommon, their recent emergence as a recognized pathogen in select marine mammal populations signals the need to identify environmental and host features in susceptible animals that predispose them to infection. Qualitative risk assessments are tools that help summarize the best available information and identify knowledge gaps for a pathogen, and aid in highlighting future areas of research. Even with high uncertainty, risk assessments can help health agencies, conservation groups, and natural resource managers develop strategies to help prevent or mitigate pathogen occurrence. Qualitative risk assessments can be updated as needed to aid in efforts to conserve highly endangered species such as southern residents, as well as inform those in the environmental and public health sectors within the One Health framework. The results of this study suggest that mucormycosis may pose an inordinately high risk to harbor porpoises (and potentially sympatric species such as southern resident killer whales) based on the detected prevalence and the severity of lesions observed at necropsy. However, the risk may be greater on an individual basis compared to the population as a whole, and may be related to other factors such as POP and trace metal burdens. Further investigations should include periodic updating of the risk assessment input data and more detailed studies of metal and contaminant trends and occurrence in apparently healthy animals.

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.

Ethics statement

Ethical review and approval was not required for the animal study because the animals included in this study were documented during routine stranding response activities conducted under the authorization of the Department of Fisheries and Oceans (Canada) and the National Oceanic and Atmospheric Administration Marine Mammal Health and Stranding Response Program (United States). The authors adhered to all guidelines for animal use provided by the governing agencies. No living animals were used for this study; samples were collected during routine necropsy of deceased animals only.

Author contributions

SN, JH, and JC conceived the project design and scope. SN, JH, DL, JG, and AS conducted data collection and submitted samples for diagnostic testing. MG and SR performed the histological examinations. LR completed fungal molecular analyses. JB conducted contaminant analyses. SN performed the risk assessment and statistical analyses. SN was responsible for the drafting of the text and received extensive comment and review by all other authors. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.962857/full#supplementary-material.

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Diversity of *Escherichia coli* found in the Salish Sea

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E. coli is a species of enteric bacteria found in the intestinal tract of humans and animals that can persist in the environment and contaminate food. Anthropogenic activity has led to pathogenic *E. coli* from humans and animals contaminating environments through the discharge of fecal wastes in sewage and agricultural runoff. While anthropogenic sources of *E. coli* have been described in terrestrial and freshwater environments, gaps remain in scientific knowledge about *E. coli* diversity in marine environments and the risk to human and animal health. This study aims to fill in some of the knowledge gaps on the diversity of *E. coli* in marine ecosystems, including: 1) describe the spatial variation of the *E. coli* sequence types (STs) found in the study region; 2) describe available information on *E. coli* STs from marine environments in terms of known relationships to determine if the isolates were related to human, animal, environment strains or novel. We analyzed a dataset of 332 *E. coli* isolates from the Salish Sea ecosystem, comprising 196 multi-locus sequence types. Sample sources included marine water near shellfish beds, marine wildlife, river otters, and a small number of marine water sites near beaches and freshwater samples from creeks into the Salish Sea. ST10 was the most frequent ST (n=12) and was found in multiple locations and sample types. For the identified STs, we searched metadata for *E. coli* STs in EnteroBase, an international *E. coli* database. Additional information on *E. coli* STs was derived from searches of published studies in PubMed. We found that diversity varied between different regions of the study area, with the greatest diversity found in an area which has partially treated wastewater outflows. A higher diversity of STs associated with animals was found in an area near were animals are raised. Many of the STs identified have been associated with virulence in humans. For a number of identified STs, no references could be found in either PubMed or EnteroBase. These findings support the importance of further studies to understand the relevance of marine *E. coli* to human and wildlife health.

KEYWORDS

***E. coli*, microbial diversity, marine mammals, EnteroBase, multilocus sequence types**

Introduction

While *E. coli* is part of the normal flora of warm-blooded mammals' intestines, it can be a significant pathogen, causing severe disease inside and outside of the intestinal tract (Centers for Disease Control and Prevention, 2014). There is tremendous genetic diversity among *E. coli*, and Multi-locus Sequencing Typing (MLST) has been used to partition *E. coli* into genetically related groups by assigning sequence types (STs) based on allelic variation within specific housekeeping genes (Ibarz Pavón and Maiden, 2009). Since its inception, MLST has been used to characterize the epidemiologic behavior of various clones of *E. coli* (Jolley et al., 2018; Vingino et al., 2021).

There are multiple routes by which *E. coli* enter water systems. Animal manure is often used for fertilizer in crop agriculture, thus contaminating vegetables and the surrounding soil (Centers for Disease Control and Prevention, 2022). Once the manure is applied to fields, bacteria can make their way to groundwater or wash into nearby bodies of water from rain, flooding, or inadequate containment of animal waste (McEwen and Collignon, 2018). The bacteria may ultimately end up in the marine environment. Wastewater treatment plants (WWTPs) are able to dump partially treated effluent, containing viable bacteria, directly into ocean waters. Raw sewage is released into the environment due to flooding, line breaks, and power outages, resulting in polluting water and environmental sources (Environmental Protection Agency, 2021). The illegal discharge of ship ballast water also contributes to bacterial contamination in marine waters with enteric bacteria, including strains of *E. coli* O157:H7 (Ruiz et al., 2000; Altug et al., 2012).

This study aims to fill in some of the knowledge gaps on the diversity of *E. coli* in marine ecosystems. The study analyzes data on previously collected isolates of *E. coli* from marine water (primarily around commercial shellfish operations) and wildlife samples while comparing the diversity of STs across four quadrants of the Salish Sea (north, south, central, and Strait of Juan de Fuca) (Norman et al., 2021; Vingino et al., 2021) (Figure 1). We simultaneously compared the STs from our dataset to metadata about associated hosts for each ST appearing in EnteroBase, a public database of *E. coli* scientific knowledge (https://enterobase.warwick.ac.uk/) and the published scientific literature in PubMed. We hypothesized that there would be variability in the diversity of the STs within *E. coli* isolated from the Salish Sea across the region, with more human-associated *E. coli* in a quadrant of the sound with less treated sewage and more animal-associated *E. coli* in a quadrant near livestock farming. Our study aim was to test this hypothesis and the objective was to look for diversity of the *E. coli* between the quadrants.

Methods

Sampling location

The Salish Sea is an international inland sea that stretches from Campbell River, British Columbia, to Olympia, Washington, encompassing the Puget Sound and San Juan Islands. The Salish Sea has a complex system of estuaries and interconnecting marine waterways. The coastline spans ~ 7,470 km. It is home to ~37 species of animals, 172 birds, and 250 fish species (The Seadoc Society, 2022). The Strait of Juan de Fuca is the primary direct access to the Pacific Ocean from the Salish Sea. According to the State of Washington Department of Ecology [Puget Sound Nutrient Source Reduction Project. Volume 1: Model Updates and Bounding Scenarios (wa.gov)], the Salish Sea area is home to about 4.5 million people (2019). Approximately 99 WWTPs line the shores of the Salish Sea. Seventy-nine WWTPs are in the United States and twenty on the Canadian side (2019). WWTPs near Vancouver and Vancouver Island, British Columbia, dump partially treated wastewater into the Salish Sea (Johannessen et al., 2015). In the counties which border the Salish Sea (Whatcom, Skagit, Snohomish, King, Pierce, Thurston, Mason, Jefferson, and Clallam), pasture and hay make up the most agricultural land use behind shellfish harvesting (Agricultural Land Use | Washington State Department of Agriculture) (See map 1).

Collection of samples

The *E. coli* isolates in this study were collected as part of previously published studies (Norman et al., 2021; Vingino et al., 2021). In those studies, five hundred fifty-one isolates of *E. coli* were obtained from various samples from the Salish Sea ecosystem. From these, 332 *E. coli* isolates were further studied with whole genome sequencing. These included isolates from marine water samples near shellfish beds (n=238), marine water samples near local Seattle beaches (n=3), freshwater samples of estuaries entering into the Salish Sea (n=5), fecal samples from river otters (*Lontra canadensis*) (n=24), rectal swabs, and fecal samples from both dead and alive seals (*Phoca vituline*) (n=53), fecal sampling from dead porpoise (*Phocoena phocoena*) (n=7), and samples isolated from English sole (*Parophrys vetulus*) (n=2) (Table S1). Differences in sampling numbers are due to the previous study (Vingino et al., 2021) assessing for antibiotic resistance in the *E. coli* isolates. While 332 isolates were sent for whole-genome sequencing (WGS) and MLST, not all were able to be sent for antibiotic resistance testing. The previous study by Vingino et al. (2021) used 305 isolates, while the current study uses all 332 whole genome sequences (Table S1).

Marine and fresh water sampling

Samples of marine water were taken in a coordinated manner by the Washington State Department of Health as part of their shellfish monitoring program (Vingino et al., 2021). To our knowledge, no samples were collected after significant rainfall or flooding events which may have influenced the amount of *E. coli* in the area. The current study does not measure the survivability of *E. coli* but rather detection by culture at the time of sampling. The *E. coli* came from the study by Vingino et al. (2021) and included marine waters samples provided by the Washington Department of Health collected from GPS-located shellfish beds as part of a fecal coliform analysis using the Environmental Protection Agency modified A-1 method (Rice et al., 2012). In addition marine water samples near beaches were collected 15 cm below the water surface. These marine water samples were diluted 1:10 (10 mL marine water and 90 mL deionized sterile water) (2021). The freshwater samples from Piper's Creek and a beaver pond adjacent to marine beaches (Golden Garden Park, Seattle, WA) were used directly and both the marine water by beaches and freshwater samples were processed using Colilert Standard Quanti-Tray2000® (IDEXX Laboratories, Westbrook, ME, USA) at the University of Washington Laboratory.

Marine mammals and river otters

Opportunistic sampling was performed on deceased harbor seals or porpoises *via* fecal swab obtained during necropsy or by rectal swab, and obtained from the study by Norman et al. (2021). Swabs were placed into Amies transport medium without charcoal, refrigerated, and shipped to a reference lab (Phoenix Laboratory, Mukilteo, WA, USA [Zoeitis References Laboratory] (Norman et al., 2021). Fecal samples from alive seals were obtained by the Washington Department of Fish and Wildlife (WDFW) from various dock sites in the Salish Sea (Vingino et al., 2021). River otter fecal sampling was collected along the Green-Duwamish River in Washington at six otter latrine locations. The samples were iced and transported to the University of Washington laboratory and processed using eosin methylene blue agar, and verified with biochemical testing (Vingino et al., 2021). More specific details have been described previously by Norman et al. (2021) and Vingino et al. (2021).

As described in the study by Vingino et al. (2021), whole-genome sequencing (WGS) was done in cooperation with the Washington Department of Health in conjunction with the FDA GenomeTrakr Project (ID 283914 - BioProject - NCBI) using Illumina (Illumina, San Diego, CA,USA) (2021). The DNA was extradite using Qiagen DNeasy Blood and Tissue Kit (Qiagen Sciences Inc. Germantown, MD USA) or MagnaPure 96 with Roche DNA and viral nucleic acid small volume kits (Roche Pharmaceuticals, Indianapolis, IND USA). From that sequence data, MLST analysis was able to be performed to determine the ST for each isolate. NCBI accession numbers for the isolates in that study have previously been described by Vingino et al. (2021). Additional NCBI accession numbers used for this study are as follows: [SAMN] 13392847, 14137889, 14214489, 15182309, 15182314, 15182317, 15344669, 15483653. [SRR] 12424353, 12643355, 12618578, 12618581, 12643343, 12643347, 12643348, 12643351, 12643352, 12643360, 12643362, 12643364, 12643366-12643368, 12643370, 12663941.

EnteroBase database search

EnteroBase version 1.1.3 is an online public and voluntary repository of scientific data regarding *E. coli*. Researchers contribute data to the database, including results of laboratory analysis and metadata, including sequence type. Other metadata for accessions include the source (animal, human or environmental), source type, source details, country of origin, and laboratory analytic results. The data are not centrally curated, so there is potential for data heterogeneity. Despite these limitations, EnteroBase data have been used for epidemiologic investigation of *E. coli* (Zhou et al., 2020).

The database was accessed on 8/11/2021 (https://enterobase.warwick.ac.uk/) to retrieve and download data for investigating the 196 STs identified in this study. A total of 153,428 entries existed in EnteroBase at the time of the query. Of the 153,428 entries, 47,319 were for the 196 STs found in this study. Entries for all 196 STs were found in the database. Information about the source (human, animal, environmental) for isolates was categorized based on available information found in EnteroBase using the listed source of the isolates: 1) found in human, animal, and environment, 2) found in animals, 3) found in humans, 4) found in the environment, 5) in animals and environment, 6) in humans and environment, and 7) in humans and animals (Table S2). STs with ten or fewer entries in EnteroBase were coded as "novel." Attention was made to any reference in the data about an ST regarding a documented marine water source, marine animals, shellfish, and fish.

We noted instances when the source niche and the source type did not match (e.g., the source niche was listed as human, but the source type entered listed as ovine/goat). Additionally, the source was not entered for every isolate. While every attempt was made to identify and mitigate these inconsistencies, some source information for isolates may be inaccurate. It is also possible that isolates in this study have been recovered from other sources but were not listed. Only a few isolates were reported by pathogen type (e.g., STEC, ExPEC, and EHEC). Thus, we may have under-identified pathogenic potential in our isolates.

Many *E. coli* STs are commensal and do not have pathogenic potential to cause disease (Centers for Disease Control and Prevention, 2014). While other *E. coli* STs are associated with one or more pathogenic types, which can cause varying degrees of illness in humans and animals (Jolley et al., 2018). The pathogenic types examined included: 1) Shiga toxin-producing *E. coli* (STEC), 2) verocytoxic *E. coli* (VTEC), and 3) enterohemorrhagic *E. coli* (EHEC), which can produce a toxin that causes various diseases with associated mortality (CDC, 2014). *E. coli* (EIEC) causes intestinal inflammation and bloody diarrhea similar to STEC (Belotserkovsky and Sansonetti, 2018). Enterotoxigenic *E. coli* (ETEC) causes diarrheal illness and is the leading cause of traveler diarrhea and illness in low-income countries, especially among children (Centers for Disease Control and Prevention, 2014). Enteroaggregative *E. coli* (EAEC) is an emerging foodborne pathogen that may cause acute or chronic diarrhea (Kaur et al., 2010), while Enteropathogenic *E. coli* (EPEC) is a common cause of diarrheal illness in children in developing countries (Ochoa and Contreras, 2011). Extraintestinal Pathogenic *E. coli* (ExPEC) can cause *E. coli* infections outside the intestinal tract, including urinary tract and bloodstream infections. It is also the second most common cause of meningitis in neonates (Russo and Johnson, 2003). Uropathogenic *E. coli* (UPEC) is a subset of extraintestinal pathogenic *E. coli*, causing only urinary tract infections (Spurbeck and Mobley, 2013). EnteroBase was queried for mention of those pathogenic types listed above. We additionally coded ST types as to whether they had been associated with STEC, EPEC, EAEC, UPEC, ExPEC, EIEC, ETEC, and EHEC strains. The number of pathogenic types for each ST was identified and added to create a "pathogen score" for a particular ST (Table S3).

Spatial distribution

ST distribution across different areas of the Salish Sea was performed using QGIS version 3.22. The Salish Sea was divided in to 4 quadrants for analysis (north, central, south and Strait of Juan de Fuca) (Figure 1). Maps were created to demonstrate the field collection sites and anthropogenic *E. coli* sources. We also used maps to give a visualization of the distribution of ST diversity. Metadata for WWTP in the Puget Sound area were obtained from the U.S. Geological Survey (USGS Water Mission Area NSDI Node). Metadata for agricultural areas were obtained from the Washington State Department of Agriculture (Agricultural Land Use | Washington State Department of Agriculture) (Figure 1).

PubMed

PubMed (PubMed (nih.gov) was searched between July and September of 2021 to determine the number of citations in the entire PubMed database for specific STs. The following search terms were utilized: ("Escherichia coli"[Mesh : NoExp] OR "E coli"[TIAB] AND ("ST (number)" OR "Sequence type (number) " OR "ST (number)"[TIAB]). The Result was categorized as follows:

0 articles found; 1-10 articles found; 11-25 articles found; 26-50 articles found; 51-100 articles found; 101-200 articles found; 201-500 articles found; 501-1000 articles found, and >1000 articles found.

Statistical analysis

Statistical analysis of *E. coli* diversity was performed using Shannon diversity index, Shannon equitable index (evenness), and Hutcheson t-test to characterize diversity of *E. coli* in the four quadrants of the Salish Sea region. The Shannon diversity index quantifies biodiversity so it can be compared to other locations or communities, but statistical significance cannot be inferred from these index values. The Hutcheson t-test is a modified version of a t-test that specifically compares the indices of the Shannon diversity index so that comparisons can be made between the diversity of two samples (Data Analytics, 2019). These statistical measures were used to test the null hypotheses 1) diversity measures of STs are similar across the four quadrants of the Puget Sound and there would be no spatial variation, 2) there are no geographic differences in diversity measures of isolates with source metadata derived from EnteroBase, Source metadata from EnteroBase were used to characterize isolates according to likely sources (human, animal, environmental). Utilizing these same tests, diversity among the quadrants for STs with pathogenic potential was also assessed. Data analysis was performed using R studio version 3.6.2.

Results

Sampling source and STs

Amongst the 332 *E. coli* isolates, 196 different STs were identified by MLST. A total of 238 isolates were obtained from marine water (72%), which included 159 STs. An additional 86 isolates with 56 different STs were from animals; seal (n=17), deceased seal (n=36), porpoise (n=7), sole (n=2), and river otter (n=24), while 33 different STs were found in animals only (Figure 1) Five isolates were from freshwater samples (ST297, ST405, ST616, ST681, ST2557), and three were from samples of marine water by beaches (ST58, ST117, ST1308). Only two STs (ST10 and ST720) were found in marine water in all four quadrants of the marine water in the Salish Sea. The most common ST found was ST10, with twelve isolates (3.6% of the total), followed by ST162, with eight isolates (2.4%). Two STs had seven isolates each (ST297, ST372). Three STs had six isolates found (ST117, ST967, and ST 1079). Most STs

identified were represented by a single isolate (n=139). The remaining (n=43) *E. coli* isolates had a total of 2-5 isolates for each ST (Table S2).

Diversity measures and statistical analysis

A high level of diversity of STs was found amongst all marine water samples in each of the four quadrants of the Salish Sea (Figure 1). The Strait of Juan de Fuca quadrant had the greatest richness of diversity among STs found there (n=51) with a Shannon diversity index (H=3.852), followed closely by the south quadrant (H=3.827). The north quadrant contained the least diversity (H=3.718). Using the Hutcheson t-test to compare the Shannon diversity index values of the Strait of Juan de Fuca and the north quadrant, there was no statistical difference in the diversity of the two quadrants (p=0.13). Of those STs found in animals and the environment, the central quadrant contained the greatest diversity (H=2.398), while the south quadrant was least diverse (H=0.95). The difference in diversity was statistically significant (p=0.0018). Of the isolates only found in the environment, the south quadrant had the highest level of diversity (H=1.7), while the north and Strait of Juan de Fuca had the least (H=1.38). There was a statistically significant higher diversity of these environmental isolates in the southern quadrant compared to the northern quadrant and Strait of Juan de Fuca (p=0.02). Of the isolates coded as novel (n=62), the Strait of Juan de Fuca contained the highest amount of diversity (H=2.75), while the south quadrant had the least diversity (H=2.39). This difference was also statistically significant (p=0.009) (Table 1). Of those STs previously described as found in humans and the environment (n=8), humans only (n=2), humans and animals (n=4), and animal only (n=2), the small sample size did not allow for statistical analysis of diversity.

PubMed and EnteroBase

Of the 196 STs found in this study, 82 (41.8%) had no published articles retrieved on a PubMed search, suggesting that these STs are rarely identified and/or reported. An additional 91 STs had only 1-10 citations available (46%). Only one ST (ST131), had greater than 1,000 articles retrieved (Figure 2). In contrast, in EnteroBase, only 47 (24%) had ¾ five entries, and 15 (7.7%) had ¾ ten entries in EnteroBase. Compared to source data from EnteroBase, 129 (65.8%) STs from this study were found in humans, animals, and the environment. Of the remaining STs, 21(10.7%) were identified in the environmental sources only, 30 (15.3%) were identified in both environmental and animal sources, eight had known sources in humans and environment, four were found in humans and animals, two were known to be found in humans only, and two were known animal sources. (Figure S1). All quadrants of the study region contained STs that were associated with animal sources only. In the northern quadrant, 84% of all STs were associated with animals (n=38), in comparison to 85% in the southern quadrant (n=41), 88% in the central quadrant (n=43), and 78% in the Strait of Juan de Fuca (n=40). STs associated with human sources were also isolated in the four quadrants, 76% (n=34) in the north, in comparison to 70% (n=34) in the central, 78% (n=38) in the south, and 86%

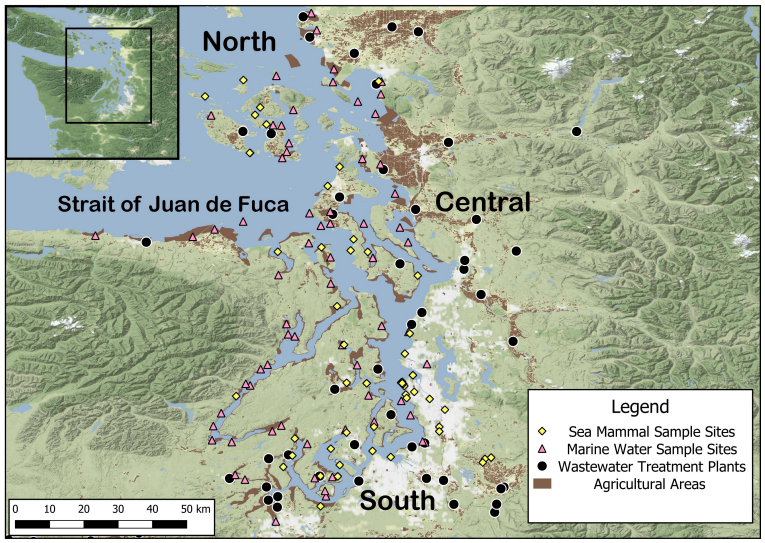

FIGURE 1
All sample locations in the Salish Sea are for water and marine mammals and anthropogenic sources.

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TABLE 1 Diversity measures for quadrants in Salish Sea.

All Marine samples	# of unique ST (richness)	Shannon Diversity Index	Evenness	Hutcheson t-test[1]
North Quadrant	45	3.718	0.977	
South Quadrant	49	3.827	0.983	
Central Quadrant	48	3.798	0.981	
Strait of Juan de Fuca	51	3.852	0.975	
				P=0.1287
Environmental and Animal				
North Quadrant	5	1.609	1	
South Quadrant	3	0.95	0.865	
Central Quadrant	11	2.398	1	
Strait of Juan de Fuca	3	1.09	1	
				P=0.0018
Environmental				
North Quadrant	4	1.386	1	
South Quadrant	6	1.7	0.816	
Central Quadrant	5	1.6	1	
Strait of Juan de Fuca	4	1.386	1	
				P=0.02
Novel				
North Quadrant	13	2.565	1	
South Quadrant	12	2.39	0.96	
Central Quadrant	14	2.615	0.991	
Strait of Juan de Fuca	16	2.75	0.993	
				P=0.009

[1]Hutcheson t-test was performed comparing the quadrants with the highest and lowest Shannon diversity index.

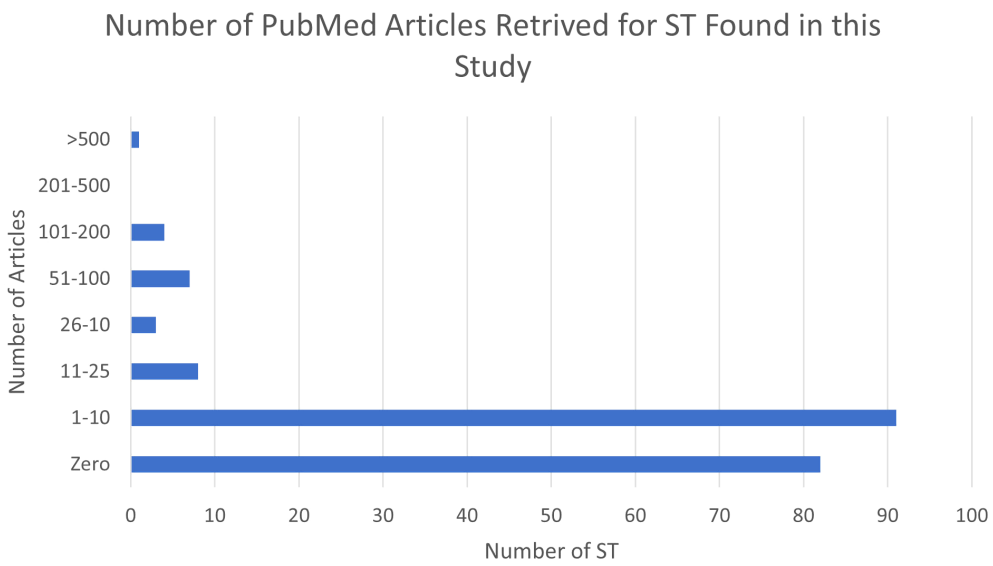

FIGURE 2
Number of articles retrieved for ST found in this study from PubMed.

(n=44) in the Strait of Juan de Fuca. For those isolates found in humans and the environment (n=8), five isolates were found in the Strait of Juan de Fuca. The two human-only isolates were obtained in dead seals. The two animal-only isolates were found in the Strait of Juan de Fuca and the southern quadrant. The four isolates found in humans and animals were found in two deceased seals, the central and the southern quadrants.

Five STs stood out when compared to known sources in EnteroBase (ST5869, ST1065, ST2164, ST9001, and ST11343) which had no previous evidence of occurrence in marine water or sea mammals. STs found in the current study were compared to available source data in EnteroBase; four isolates STs were found in marine water; twenty-two isolates STs were found in marine fish, eleven STs in marine mammals, and forty STs in shellfish (Table 2).

Information on pathogenic types according to EnteroBase

The 196 STs found in this study were queried in EnteroBase to determine if there were known STEC/VTEC, EPEC, EAEC, ExPEC, EIEC, ETEC, EHEC, or UPEC isolates associated with these STs. Thirty-five of the STs found in our study were UPEC strains. ST10 was the most abundant ST found in this study (n=12) and was associated with STEC/VTEC, EPEC, EAEC, ExPEC, and ETEC strains. Six STs (ST10, ST58, ST101, ST155, ST297, ST1248) were associated with STEC strains, ten EPEC strains, three EIEC strains, 19 EAEC, 28 ExPEC strains, seven ETEC, and three EHEC (Table S3). In those STs with at least one pathogen type, the south quadrant contained the highest diversity (H=2.955), while the central quadrant contained the least diversity (H=2.58). All quadrants sampled for marine water, freshwater, seals (alive and deceased), porpoise, and sole had at least one pathogen associated ST strain. EnteroBase was queried for pathogenic type STs with sources found in marine mammals and shellfish, with known disease-causing strains. There were 21 STs with pathogenic potential found in shellfish and 8 STs found in marine animals with pathogenic potential (Table S3).

Discussion

This study describes a high degree of *E. coli* ST diversity in a marine environment as evidenced by Shannon diversity index scores of >3 in the marine water samples from the four quadrants of the Salish Sea. Five of 62 novel STs [5869, 1065, 11343, 9001, 2164] have not previously been reported in marine and animal environments in published literature. Within the quadrants, there was a high degree of diversity in *E. coli* STs, including novel isolates, those with known environmental sources, environmental and/or animal sources. There was not a high number of isolates of human origin in the northern quadrant which was surprising given the outflow of partially treated sewage. There was higher diversity of STs associated with animals in the central quadrant where there was proximity to animal agriculture. Evenness across the four quadrants was consistently close to one. This finding is not surprising given the high proportion of identified STs represented by one isolate; thus, there is a similar abundance, suggesting there is no dominant strain present in the quadrants at the sampling time. There was no concentrated variation in the distribution of STs. ST10 was the most abundant isolate found (n=12), and it was isolated in all four quadrants of the marine water and in a river otter *E. coli*.

Our findings of high diversity of *E. coli* in a marine environment are consistent with published studies of freshwater environments that have reported higher *E. coli* diversity utilizing the Shannon diversity index. A high degree of *E. coli* diversity was found in freshwater wells in Nigeria, which the authors speculated was due to multiple sources of contamination, including proximity to septic tanks and erosion (Odetoyin et al., 2022). Chandran and Mazumder (2015) described a high level of *E. coli* diversity, with seasonal variations, within a freshwater lake on Vancouver Island,

TABLE 2 ST found in this study that had a marine source in EnteroBase.

Marine Mammal[a]	Shellfish[b]	Fish (specific saltwater)[c]	Fish (non-specific)	Salt/Marine water
ST: 10, 38, 69, 75, 127, 131, 132, 155, 162, 491, 4219	ST: 10, 38, 48, 58, 69, 73, 101, 117, 127, 155, 162, 206, 215, 224, 297, 327, 345, 349, 372, 446, 540, 547, 641, 666, 681, 906, 942, 1049, 1056, 1079, 1304, 1423, 1611, 2144, 2521, 3601, 4038, 4162, 4481, 6096, 6188	ST: 132, 212, 327, 1704, 1706,	ST: 10, 12, 38, 48, 46, 58, 101, 109, 127,131, 162, 224, 362, 746, 1629, 2522, 6998	ST: 38, 40, 46, 130

[a]Seal, dolphins, sealions.
[b]Oysters, mussels, bi-valves, shrimp, crab, mollusk.
[c]Sea bream, tuna, sardine, salmon.

British Columbia, Canada. The authors had previously described greater *E. coli* diversity in animal and avian hosts than in human hosts (Chandran and Mazumder, 2013; Chandran and Mazumder, 2014). They hypothesized that the high level of diversity and seasonality variation of *E. coli* in the 2015 study was due to the variations in animal populations in the area as the lake was in a forested watershed. This lake is located near the Strait of Georgia, which connects to the north quadrant of the Salish Sea. The Salish Sea is home to multiple species of animals and birds that depend on the marine ecosystem (SeaDoc, 2021). This may be one reason for the overall high diversity and differences in diversity in the quadrants when comparing isolates with known sources of animal and environment and environmental-only isolates.

In the current study, isolates derived from water samples from the Strait of Juan de Fuca had the greatest ST richness. Five of the eight isolates previously associated with humans and the environment were also sampled in this region. The Strait of Juan de Fuca had the highest Shannon diversity index score, followed very closely by the southern quadrant, however, there was no statistical significance in the amount of diversity between the Strait and the quadrant with the lowest score (north). Interestingly, the Strait of Juan de Fuca also had the most diversity of novel STs as compared to the other quadrants. Though it is important to note the other quadrants had high diversity with index scores greater than two when assessing the biodiversity for the novel isolates. The northern quadrant had the fewest total number of isolates identified and the diversity did not differ significantly from the other quadrants for any of the categories investigated. This is somewhat surprising due to the presence of multiple untreated sewage outfalls in Vancouver and Victoria, British Columbia (Krogh et al., 2017). We had hypothesized due to the presence of untreated sewage, there would be a higher level of diversity and more human isolates in this quadrant. One possible explanation is that tidal currents and winds can carry the sewage westward into the Strait of Juan de Fuca, which may account for the variation found in this region (Krogh et al., 2018).

The central quadrant had the most diversity in STs found in animals and the environment, and this difference was statistically significant compared to diversity in the other quadrants. This quadrant had the second-highest diversity index score for environmental isolates. Isolates of STs with known pathogenic potential were found in all sample types analyzed, though the greatest diversity was found in samples from the southern quadrant of the Salish Sea. In those STs with known environmental sources, the highest diversity was in the southern quadrant, followed closely by the central quadrant. It is unclear why the southern quadrant had the most diversity. The diversity of STs may be influenced by the density of marine animal populations found in the Vingino et al. study (2021) and the presence of WWTPs in the southern quadrant, similar to the findings of Chandran and Mazumder (2015).

In addition to the high diversity of *E. coli* STs in this study, there were also interesting findings in regard to the novel STs. For example, *E. coli* ST5869, found in marine water, had entries in EnteroBase listing human and animal sources, including animals from China, Belgium (Brussels), and the United States (Tennessee), human sources from Cambodia and the Netherlands, and an avian source in Kenya. Scholarly articles have previously described this ST as being found in food sources in Germany and Belgium (Pauly et al., 2021; Garcia-Graells et al., 2020), animals treated in a veterinary clinic in Switzerland (Schmitt et al., 2021), and humans in Nigeria (Jesumirhewe et al., 2020). We could not find a previous report of this ST in a marine environment.

While *E. coli* ST1065 and ST11343 were associated only with human sources in EnteroBase, they were found in necropsy samples from dead seals in the current study. In EnteroBase, ST1065 was mentioned to have human source in an isolate from France and from the United States (Washington). In the expanded literature review, one article linked ST1065 to cattle (Isiko et al., 2015). We did not find any previous case reports of this ST in a marine animal. No scholarly articles were found regarding ST11343, even in an expanded search from multiple databases. While this ST was mentioned in EnteroBase as being isolated from human sources, mention of its occurrence in animals has apparently not been reported previously.

E. coli ST9001, isolated from a live seal in the current study, was referenced as human and environment in EnteroBase with sources from North America (not further specified) from humans and water. *E. coli* ST2164 isolated from a river otter in the current study was referenced in the environment only in EnteroBase with sources from Canada and Massachusetts and water sources from Florida and Washington. Neither *E. coli* ST9001 nor *E. coli* ST2164 were found in an expanded literature search in multiple databases. We did not find previous reports of these STs in animals.

There were several strengths of this study including how sampling of animals were done. For example most of the animals were from deceased marine animals which was opportunistic. In addition there was no human contact with the otters and alive seals while collecting fecal samples (Vingino et al., 2021), thus there was no harm or stress caused to marine mammals during the collection of samples. Although the lack of human isolates for comparison, the current study expanded our knowledge using a large collection of *E. coli* isolates from the Salish Sea including water and animal samples.

This study had several limitations. The EnteroBase is a public database not centrally curated and, as such, relies on the users to enter accurate information. In addition, STs, especially those seeming to be novel, may be underreported as the database relies on human entry and is not auto populated by lab detection software. The literature review and counting of articles associated with each ST were performed with PubMed only. Additional articles may be found on different scholarly

publication databases. This study aimed not to search multiple databases for each ST, but to give a broad overview of available literature in an extensive, primarily peer-reviewed database associated with each. Of note, an expanded literature review utilizing multiple databases was performed for *E. coli* with ST5869, ST1065, ST11343, ST9001, and ST2164 as described above to identify whether our reporting was novel. Finally, because this study utilized a previously collected dataset, the assumption is made that the data contained in the dataset is accurate and without errors. Samples for dead seals and porpoises collected for the original dataset were opportunistic and taken postmortem.

Conclusion

To our knowledge, this study is the most extensive characterization of *E. coli* diversity in a marine environment to date. The diversity found may be due to many changes in the Salish Sea and surrounding environment but determining these factors was beyond the scope of the current project. However, even given these limitations, the study can serve as a blueprint for future studies of *E. coli* in marine ecosystems including both the water and the animals living in and near marine environments around the world.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Genome Trakr Project ID 283914-BioProject-NCBI.

Author contributions

JG did all the literature searches, data analysis and writing. MR, PR, and SW were involved with the original paper that generated the data set, the direction of the study and in the writing of the current manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.967435/full#supplementary-material

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Poor pulmonary health in Barataria Bay dolphins in the eight years following the *Deepwater Horizon* oil spill

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The *Deepwater Horizon* (DWH) disaster resulted in large-scale contamination of bays, sounds, and estuaries in the northern Gulf of Mexico, home to multiple stocks of common bottlenose dolphins (Tursiops truncatus). Inhalation, aspiration, ingestion, and dermal absorption of oil and its toxic components were all considered possible routes of exposure for dolphins living within the oil spill footprint. To determine if dolphins were adversely impacted, catch-and-release health assessments were performed in heavily-oiled Barataria Bay (BB), Louisiana, and in Sarasota Bay (SB), Florida, a comparison site with no DWH oil contamination. Initial studies were conducted as part of a Natural Resource Damage Assessment (2011-2014) and follow-on studies were performed between 2016-2018 with support from the Gulf of Mexico Research Initiative. Ultrasound was used to evaluate the dolphins' pulmonary health, including the presence/absence of pleural effusion, nodules, masses, consolidation, and alveolar interstitial syndrome (AIS). When present, AIS was further graded by severity (mild, moderate, or severe) and distribution. Based on the presence and severity of abnormalities, each dolphin was given an overall lung disease score (normal, mild, moderate, or severe). Normal to mild scores were considered within expected limits for a wild population, therefore the prevalence of normal-mild versus moderate-severe scores was compared between the oiled and unoiled sites. Separate analyses were conducted for dolphins alive in 2010 (and in BB, presumably exposed to DWH oil), and those born after 2010. For the dolphins alive in 2010, temporal trends were also examined using generalized additive models (GAMs). Results showed a strong difference (p=0.000357) in moderate to severe lung disease between the two

sites for dolphins alive in 2010, but no significant difference (p=0.6745) between the sites for dolphins born after 2010. In BB dolphins, the prevalence of moderate to severe lung disease did not decrease in the years after the spill, and in fact, potentially worsened (p=0.0644 for trend over years), with the highest prevalence (0.61) being in 2018. Moderate to severe AIS remained a persistent finding in BB dolphins, and several animals had a pattern of AIS that was more severe ventrally than dorsally, with evidence of chronic, progressive disease states.

KEYWORDS

Deepwater Horizon, oil spill, dolphin, pulmonary, health

Introduction

In April 2010, the *Deepwater Horizon* (DWH) offshore drilling rig exploded and sank, resulting in large-scale release of oil that contaminated bays, sounds, and estuaries in the northern Gulf of Mexico (NGoM) (Michel et al., 2013). More than 3 million barrels of oil (~900 million pounds) were released before the well was sealed (U.S. v. BP et al., 2015), resulting in an oil slick that spanned ~43,000 square miles of water and oiled more than 1,000 miles of shoreline habitats (Michel et al., 2013; ERMA, 2015). Although clean-up efforts removed ~600 million pounds of oil-contaminated waste from Gulf waters and nearshore habitats of the NGoM (EPA, 2011), the oil spill caused substantial injury to marine life (Deepwater Horizon Natural Resource Damage Assessment (NRDA) Trustees, 2016).

As DWH oil spread throughout the NGoM, multiple cetacean species were observed in the oiled areas (Aichinger Dias et al., 2017). Response monitoring activities from April through September 2010 documented more than 1,100 individuals of 10 different cetacean species swimming through thick surface oil or surface oil sheen (Figure 1) (Aichinger Dias et al., 2017; Wilkin et al., 2017). Cetaceans living within the oil spill footprint were likely exposed through multiple routes, including inhalation of volatile organic compounds, direct aspiration of oil droplets, ingestion with or without subsequent aspiration, and dermal absorption of oil and its toxic components (Takeshita et al., 2017). Further, oral ingestion may have occurred through incidental ingestion of oil on the surface of the water and/or suspended in the water column, or through the ingestion of contaminated prey and/or sediments during foraging activities (Godard-Codding and Collier, 2018; Quigley et al., 2022).

In the months that followed, one of the largest and longest-lasting marine mammal Unusual Mortality Events (UMEs) documented in the NGoM began and continued through 2014 (Litz et al., 2014). Marine mammal stranding network responders collected biological data and samples from carcasses, and after a comprehensive investigation into all potential causes of the mortality event, the most likely cause was determined to be the DWH oil spill (Venn-Watson et al., 2015a). Some of the most consistent necropsy findings within the oil spill footprint were bronchopneumonia and adrenal gland atrophy in non-perinate dolphins and an increased prevalence of fetal distress and *in utero* pneumonia in dead perinates (Venn-Watson et al., 2015b; Colegrove et al., 2016).

While the marine mammal UME investigation was underway, a Natural Resource Damage Assessment (NRDA) was being conducted to assess the potential impact of the DWH oil spill on the NGoM ecosystem, including marine mammals. Live bottlenose dolphins (*Tursiops truncatus*) living in heavily-oiled Barataria Bay (BB), Louisiana, and oil-impacted Mississippi Sound (MS), Mississippi/Alabama, received comprehensive health exams during catch-and-release field studies. Live dolphins were also examined in Sarasota Bay (SB), Florida, a comparison site with no DWH oil contamination. Dolphins in the heavily-oiled sites had multiple health issues, including moderate to severe lung disease, poor body condition, an impaired stress response, and hematological/serum chemistry indicators of inflammation, hypoglycemia, and abnormal iron levels (Schwacke et al., 2014). During 2011-2013, nearly half of the dolphins evaluated by experienced marine mammal veterinarians in oil-impacted habitats (BB and MS) were considered unhealthy, indicated by a guarded or worse prognosis, and 17% percent of examined dolphins received a poor or grave prognosis, meaning they were not expected to survive (Schwacke et al., 2014).

The increased prevalence of dolphins with compromised health coincided with high mortality rates within the oil spill footprint. Follow-up studies of BB dolphins using mark-recapture survival models yielded estimated annual mortality rates of 13.2-19.6% in the years immediately following the spill (Lane et al., 2015; McDonald et al., 2017), which were much

FIGURE 1
Bottlenose dolphin surfacing for a breath within the DWH oil spill footprint, with oil adhered to its head and dorsal fin (photo: NOAA, July 2010).

higher than mortality rates previously reported for bottlenose dolphins using similar techniques near Charleston, South Carolina (4.9%), and Sarasota, Florida (3.8%) (Wells and Scott, 1990; Speakman et al., 2010). Alternative hypotheses were considered, including exposure to harmful algal blooms (Deepwater Horizon Natural Resource Damage Assessment (NRDA) Trustees, 2016), persistent organic pollutants (Balmer et al., 2015), and infectious disease outbreaks (Venn-Watson et al., 2015a). These factors were ruled out as likely contributors to the increased mortality rates, and exposure to toxic oil components was determined to be the most likely cause of increased mortality within the DWH oil spill footprint (Deepwater Horizon Natural Resource Damage Assessment (NRDA) Trustees, 2016; Takeshita et al., 2017).

In the year following the DWH spill, dolphins examined within the oil spill footprint had an approximately 5-fold higher prevalence of moderate to severe lung disease compared to dolphins living outside the oiled region, based on pulmonary ultrasound examination (0.34 in BB versus 0.07 in SB, the comparison site) (Schwacke et al., 2014). During 2013 and 2014, the prevalence of moderate to severe lung disease among BB dolphins decreased slightly (0.23 and 0.25, respectively), but remained elevated relative to the prevalence at the SB non-oiled comparison site (Smith et al., 2017). Concurrent pathology

investigations of dead dolphins recovered from the NGoM in the years following the spill documented similar pulmonary findings (Venn-Watson et al., 2015b). The prevalence of bacterial pneumonias, many severe, in carcasses recovered within the oil spill footprint was significantly higher than in comparison populations [0.22 (oiled) vs 0.02 (non-oiled)]. Pneumonias were caused by multiple bacterial pathogens, indicating that lung disease was not due to infection with a single, highly pathogenic bacterium, and may have been due to secondary infection of damaged lung tissue (Venn-Watson et al., 2015b).

The increased prevalence of lung disease in dolphins living within the DWH oil spill footprint was consistent with respiratory findings from humans exposed to oil and its toxic components (Zock et al., 2007; Jung et al., 2013; Alexander et al., 2018; Gam et al., 2018a; Gam et al., 2018b). However, relatively few studies have focused on the chronicity of respiratory disease post-exposure (Zock et al., 2012; Zock et al., 2014; Lawrence et al., 2020). Here we combined these previously reported data with additional data collected from 2016-2018 as part of a Gulf of Mexico Research Initiative study to examine temporal trends and assess if pulmonary disease persisted in wild dolphins as a chronic effect of the DWH oil spill.

Materials and methods

Dolphin catch-and-release health assessments

Dolphin health assessments were conducted in BB in 2016, 2017, and 2018, as well as SB (a non-oiled comparison site) in 2012 and 2015- 2018. For comparative analyses over time, we included previously reported pulmonary data from health assessments in BB (2011, 2013, 2014) and health assessments in SB (2011, 2013, 2014) (Schwacke et al., 2014; Smith et al., 2017). The SB 2012 health assessments were not previously included in the analysis because they were not part of the DWH NRDA. For the present study, we included the SB 2012 results to provide a more comprehensive analysis over time. Standardized, catch-and-release methodologies and diagnostic sampling techniques are described elsewhere and were similar during all study years and across locations (Wells et al., 2004; Schwacke et al., 2014). Briefly, dolphins were encircled with a seine net and supported by experienced handlers for examination. Small calves with a known or suspected age of less than two years were avoided. The amount of time each animal was restrained and the time spent out of the water were minimized (less than an hour), and veterinary staff continuously monitored animals throughout their health assessments.

Live dolphin health assessments in Barataria Bay were conducted under NMFS permit numbers 932-1905/MA-009526 and 18786 issued to the NMFS Marine Mammal Health and Stranding Response Program. All animal procedures were reviewed and approved by NMFS's Institutional Animal Care and Use Committee. Sarasota Bay health assessments were performed under National Marine Fisheries Service Scientific Research Permit Nos. 15543 and 20455 approved annually by Mote Marine Laboratory's Institutional Animal Care and Use Committee. All animal research was conducted in accordance with ARRIVE guidelines 2.0 (Percie du Sert et al., 2020).

Pulmonary ultrasound evaluations

To specifically evaluate pulmonary health, we utilized thoracic ultrasound techniques developed for U.S. Navy dolphins (Smith et al., 2012) and applied them to wild dolphin health exams. The dolphin body is well-suited for ultrasound, as their skin is smooth and hairless and doesn't require any preparation. Portable ultrasound machines are powerful enough to penetrate dolphin blubber and rugged enough to be used in extreme field conditions (e.g. high air temperatures, saltwater environments, and unstable operating platforms). Therefore, we utilized portable Sonosite Edge® ultrasound machines (Sonosite, Bothell, Washington 98021, USA) and curvilinear 2-5MHz abdominal transducers to conduct pulmonary health evaluations.

Ultrasound exams were performed by experienced marine mammal sonographers (CRS, FMG, JMM) using the dorsal-ventral slide technique as previously described (Smith et al., 2012). Pulmonary abnormalities were detected following standardized methods (Smith et al., 2012; Schwacke et al., 2014) and divided into previously defined categories: (1) pleural effusion, or fluid surrounding the lungs; (2) superficial pulmonary nodules, or <2cm round/ovoid foci of non-aerated lung; (3) pulmonary masses, or ≥2cm well-defined areas of non-aerated lung; (4) alveolar-interstitial syndrome, or reduced air in the lung and replacement of air with cellular infiltrates or fluid, and (5) pulmonary consolidation, where fluid and/or cellular infiltrates completely occupy some portion of the lung. Alveolar-interstitial syndrome (AIS) was graded as mild, moderate, or severe as follows: mild – occasional clusters of ring-down artifacts; moderate – frequent clusters of ring-down artifacts, distributed throughout the dorsal, ventral, or entire lung field; severe – contiguous ring-down artifacts that created a 'white-out' effect and loss of reverberation artifact, detected in multiple areas. When present, distribution of AIS throughout the lung field was noted, such as a generalized distribution throughout the lung or localized to a specific area in the lung. Additionally, if AIS was more severe as the sonographer slid from dorsal lung to ventral lung, it was noted as 'AIS worse D-V'. Ultrasound artifact identification was based on a large body of medical literature, as reviewed by Baad et al. (2017). Pulmonary nodules (<2cm) and masses (≥2cm) were measured and described.

Once examinations were complete, each lung (left and right) was given an overall score: normal (no evidence of disease), mild,

moderate, or severe lung disease (Smith et al., 2017). Following the approach of Schwacke et al., (2014) and Smith et al. (2017), a binary variable was created to indicate lung scores that were normal-mild, which are within expected limits for a wild dolphin, versus moderate-severe disease.

Classification of age cohorts

In order to compare lung disease in BB dolphins presumably exposed to DWH oiling in 2010 versus those that were born after the spill, individual dolphins were classified as alive in 2010 (pre-2010 cohort), or born after 2010 (post-2010 cohort) following methods described by Schwacke et al. (2022). While age estimates from dental radiography and dentinal growth layer analysis were available for many of the sampled dolphins, there is uncertainty associated with these estimates (Herrman et al., 2020). Therefore, we chose to rely on observational life history evidence from photographic monitoring to obtain definitive classifications. Briefly, dorsal fin images of sampled dolphins were compared to photographic-identification (photo-ID) catalogs developed from long-term, small vessel studies in BB (2010-present) and SB (1970-present) (Wells and Scott, 1990; McDonald et al., 2017). Photo-ID surveys in SB were conducted regularly (monthly since 1992) and the vast majority of dolphins sampled in SB were known to the researchers prior to their health assessment. Those that had been observed in 2010 or earlier were classified as pre-2010; those observed as neonates in 2011 or later were classified as post-2010. Photo-ID surveys in BB were not initiated until 2010. For BB, a highly experienced photo-ID researcher (TS) searched the catalog for all sightings of the sampled dolphins prior to their health assessment. Dolphins that were sighted in 2010 were classified as pre-2010; dolphins sighted in 2011 or 2012 and deemed to be a non-calf (> 2 years) based on size at the time of the sighting were also classified as pre-2010. Dolphins observed as neonates in 2011, or in 2012 and later as a neonate or small calf were classified as post-2010. Cohort assignment could not be definitely determined for the remaining BB dolphins.

Statistical analysis

The prevalence of moderate-severe lung disease was compared between study sites separately for the pre-2010 and post-2010 cohorts. Right lungs were not routinely evaluated between 2011-2013 (due to health assessment time constraints), therefore only left lung scores were used for statistical analyses to facilitate comparison across years. For the pre-2010 cohort, we used a generalized additive model (GAM) with binomial response variable (normal or mild vs moderate or severe disease) as a function of study site (factor covariate BB or SB), year (continuous covariate), and sex (factor covariate male or female). A logit link function was used, and year was modeled using a thin plate regression spline with basis dimension k=3. We fit multiple models, including models with the main effects and an interaction of year and site, and then used Akaike Information Criteria (AIC) value to select the most parsimonious model.

Due to the short time span since the spill and the fact that very young dolphins (< 2 years old) were avoided for sampling, the number of post-2010 was limited; therefore, we did not attempt a temporal analysis (i.e., we did not include year as a covariate). This left only factor covariates, meaning models for the post-2010 cohort were generalized linear models (GLMs) rather than GAMs.

To visualize the prevalence of various lung abnormalities that contributed to the lung disease scores, heatmaps were generated and stratified by study site and age cohort. Nodules, pleural effusion, consolidation, masses, and AIS worse D-V were treated as binary variables (absent=0; present=1), with prevalence calculated as the proportion of dolphins for which the abnormality was present. In order to visually capture the gradient of severity of AIS scores, dolphins with no or mild AIS were assigned a 0, dolphins with moderate AIS were assigned 0.5, and 1.0 for severe AIS. Note that this assignment infers that severe scores were twice as bad as moderate scores, which is not necessarily accurate; however, the numeric assignment was only used for visualization purposes, not for statistical inference.

To determine whether the prevalence of the AIS worse D-V pattern changed over time for pre-2010 BB dolphins, an additional GAM was conducted, again with year modeled using a thin plate regression spline with basis dimension 3. All analyses were conducted in R (R Core Team, 2021); we used the mgcv package for GAM analyses (Wood, 2017) and the glm function within the R stats package. The gam.check function from the mgcv package was used to confirm convergence and check basis dimension results to ensure that k was not too low.

Results

Between 2011 and 2018, we evaluated the pulmonary health of 171 BB dolphins: 132 were alive at the time of the spill (2010) and 18 were born post-spill. Age cohort classification could not be determined for 21 BB individuals. Over the same time period, we assessed the pulmonary health of 103 dolphins from the unoiled comparison site (SB): 85 were alive at the time of the spill (2010) and 18 were born post-spill.

Dolphins alive in 2010 during the DWH spill

In the first year post-spill (2011), the prevalence of moderate to severe lung disease was 0.34 in BB dolphins (Figure 2A). The

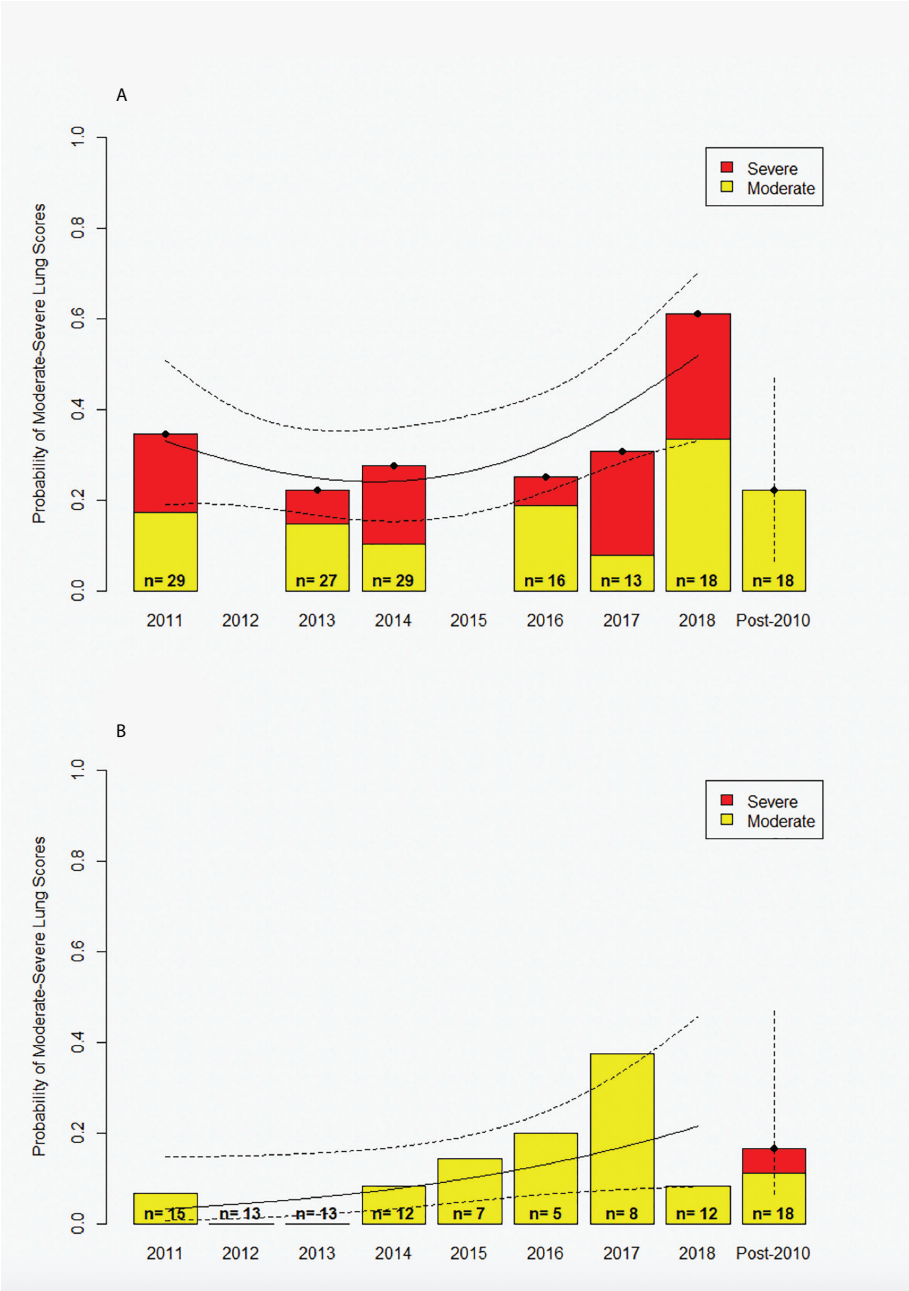

FIGURE 2
Probability of moderate and severe lung scores for **(A)** Barataria Bay and **(B)** Sarasota Bay. Probability for dolphins alive at the time of the spill is stratified by year (2011-2018); lung scores for dolphins born after 2010 were pooled within each site due to limited samples sizes ('Post-2010'). Red and yellow portion of bars represent proportion of severe and moderate scores, respectively. Fitted curves (solid) and 95% CIs (dashed curves) are from the Generalized Additive Model (GAM) with separate smoothing splines for sampling year (Barataria Bay p=0.0644, Sarasota Bay p=0.0655). Vertical dashed lines for post-2010 cohorts represent 95% binomial confidence intervals.

lowest prevalence in BB was observed in 2013 (0.22), but prevalence had increased again by 2018 (0.61). In SB, no severe lung disease was observed and the prevalence of moderate lung disease was low (0.00-0.08) from 2011-2014, but increased between 2015-2018 (0.14-0.38) and returned to 0.08 in 2018. We note that there is significant uncertainty in the higher SB estimates (2015-2018) due to very low sample sizes (2015 N=7, 2016 N=5, 2017 N=8; Figure 2B).

Of the GAMs tested, the model with site and year covariates that had the lowest AIC value was selected for

further inference (Table 1). Study site was strongly associated with the prevalence of moderate to severe lung disease (p=0.000357), with dolphins sampled in heavily-oiled BB having the higher prevalence. The GAM provided some evidence that prevalence of moderate to severe lung disease changed across years (p=0.0644 for BB, p=0.0655 for SB). However, in BB the prevalence did not consistently decrease over time after the spill (Figure 2A), and in fact the highest prevalence measured was in the final year of sampling.

AIS was a substantial factor in the BB lung disease cases, and in some individual dolphins was more severe ventrally than dorsally (AIS worse D-V). This spatial distribution pattern of AIS in the lung was not seen in any of the SB dolphins sampled. The pattern was observed in BB dolphins alive at the time of the spill, and applying a GAM to examine the prevalence of the condition over time indicated an increase over the years (p=0.0002). The prevalence of this pattern was also highest in the final year of sampling (Figure 3). Other thoracic abnormalities included pleural effusion, pulmonary nodules, consolidation, and masses in BB (Figure 4A). Some of these abnormalities, including pulmonary nodules, were also seen in SB, but to a much lesser extent (Figure 4B).

Dolphins born after the DWH spill

For dolphins born after the spill, the GLM with only site as a factor had the lowest AIC (Table 1) and was selected for further inference. Prevalence of moderate or severe lung scores did not differ between BB and SB (p=0.6745), and was relatively low (Figure 2) for this younger cohort. Pulmonary nodules were the most commonly observed abnormality for both BB and SB cohorts (Figure 5).

Discussion

In the aftermath of the DWH disaster and subsequent oiling of estuarine and coastal habitats, a high prevalence of moderate to severe lung disease was found in bottlenose dolphins living within Barataria Bay, one of the heaviest oiled estuaries. BB dolphins have high site fidelity, meaning that animals tend to live in the same region for multiple years and are unlikely to leave (Lane et al., 2015; Wells et al., 2017). Our results determined that dolphin pulmonary health has not improved for BB dolphins alive during the spill. The high prevalence of moderate to severe lung disease years following the spill suggests that the lung damage that occurred is chronic and likely progressive, which should be considered when investigating potential pathways and mechanisms of respiratory compromise. Other conditions that possibly contributed to the chronic lung disease included an increased susceptibility to lung infections due to oil-induced immune system aberrations (De Guise et al., 2021), cardiac damage with secondary pulmonary compromise (Linnehan et al., 2021), and/or age-related health changes.

In bottlenose dolphins and other cetaceans, the lungs serve a dual purpose of both respiration and buoyancy control (Ridgway et al., 1969). Investigations of dolphin pulmonary anatomy have shown that terminal airways are reinforced with cartilage, and myoelastic sphincters surround terminal bronchioles and alveolar entrances (Simpson & Gardner, 1972). The alveoli can completely collapse at depth, forcing air into the reinforced air spaces, presumably for prevention of decompression sickness (Ridgway et al., 1969). The presence of moderate to severe pulmonary disease would be expected to impair these physiologic mechanisms, negatively impact buoyancy, and increase energetic demands. This is supported by the authors'

TABLE 1 Results from the generalized additive model analysis (pre-2010 cohorts) and generalized linear model analysis (post-2010 cohorts) of overall lung scores.

		p-values				
Model	**AIC**	**Site**	**Sex**	**Site*Sex**	**Year : BB**	**Year : SB**
Pre-2010 Cohorts						
Site	223.65	0.000215	–	–	–	–
Site + Year by Site	**218.99**	**0.000357**	-	-	**0.0644**	**0.0655**
Site*Sex	227.07	0.00622	0.46162	0.85426	–	–
Site + Sex + Year by Site	219.87	0.000517	0.315939	–	0.0448	0.0752
Site * Sex + Year by Site	221.43	0.00294	0.23935	0.52658	0.0395	0.0654
Post-2010 Cohorts						
Site	**39.290**	**0.6745**	-	-	-	-
Site+Sex	40.537	0.564	0.381	–	–	–
Site*Sex	41.201	0.224	0.154	0.257	–	–

Final models (bolded) were selected based on lowest Akaike Information Criteria (AIC).

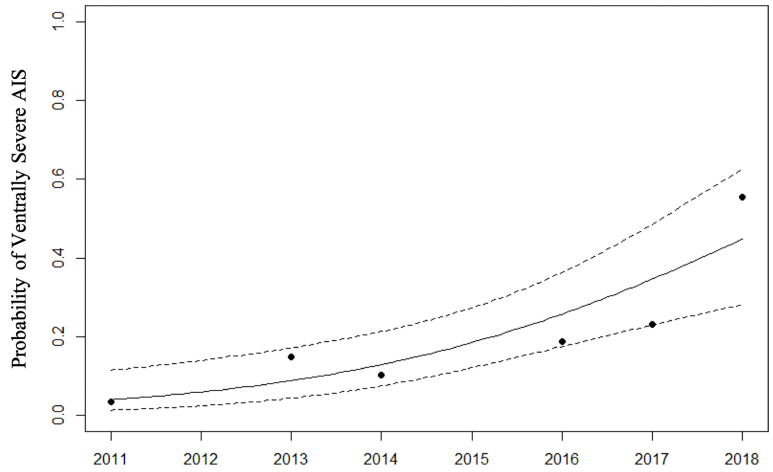

FIGURE 3
Probability of ventrally severe alveolar interstitial syndrome (AIS) in BB dolphins over years. Solid and dashed lines represent GAM model fit and 95% confidence intervals. p-value for smooth term < 0.001.

(CRS, FMG, FIT, JMM, MI, RSW) personal observations of dolphins in human care diagnosed with moderate to severe pulmonary disease that have altered swim and dive patterns, likely due to decreased functional lung capacity and concomitant impaired buoyancy.

The physiological adaptions of dolphin lungs that support diving and swimming in marine ecosystems may make these species more susceptible than humans and other air-breathing animals to the negative impacts associated with inhaled or aspirated contaminants. Cetaceans breathe at the air-water interface, where high levels of volatile compounds and oil-containing aerosols and droplets were released from the DWH oil slick (de Gouw et al., 2011; Ryerson et al., 2012). Adult bottlenose dolphins take rapid breaths that begin with an explosive exhalation and are followed by deep inhalation of ~10 liters of surrounding air into their lungs, all in less than a second (Ridgway et al., 1969; Piscitelli et al., 2013). This air intake goes directly from the blowhole to the dolphin's lungs, without protective cilia or nasal turbinates to filter the air. Dolphins often hold their breath for several minutes while swimming, foraging, and interacting with others, before returning to the surface of the water to take several breaths. During these respiratory cycles, dolphins exchange up to 90% of deep lung air (Ridgway et al., 1969), which could allow contaminants ample time and opportunity to impact the lung tissue.

A potential route of oil exposure in marine mammals is aspiration of liquid oil from the surface of the water and/or aerosolized oil droplets (Takeshita et al., 2017). During the DWH oil spill event, dolphins were seen surfacing in oil slicks and others were documented with crude oil on their heads (Figure 1) (Schwacke et al., 2014). In animals, aspiration of petroleum products (e.g. kerosene, crude oil) can cause direct injury to lung tissue or lead to aspiration pneumonia (Coppock et al., 2012; Mostrom, 2021). In dolphins, active infectious pneumonia of varying severity has been well documented (Smith et al., 2012). Less frequently, aspiration pneumonia has been diagnosed and managed (CRS, FMG, JMM unpublished). For animals in human care, clinical evidence typically includes elevated blood-based markers of systemic inflammation, confirmation of pneumonia on radiography and/or computed tomography, isolation of infectious agents from respiratory tract sampling, and a positive response to therapy. When treatment is effective, complete or near-complete resolution of the pulmonary abnormalities can occur. In wild dolphins with no access to medical treatment, aspiration pneumonia could be fatal, and if survived, could plausibly lead to chronic, pulmonary disease.

Moderate to severe AIS remains a persistent finding in BB dolphins (2011-2018). Possible causes include pneumonia, pulmonary edema, and pulmonary fibrosis (Smith et al., 2012). During the initial health assessments of BB dolphins (2011), infectious pneumonia was considered the most likely cause of AIS based on associated ultrasound findings (such as consolidation) and blood-based evidence of inflammation, anemia, and possible septicemia (Schwacke et al., 2014). In subsequent years (2013-2014), the animals' overall health status stabilized somewhat, and chronic, pulmonary diseases were considered more likely than active, infectious pneumonias (Smith et al., 2017). From 2016-2018, the clinical picture slightly deteriorated, with increasing prevalence of severe lung disease. Several of the BB dolphins had a pattern of AIS that was more severe ventrally than dorsally (2016-2018).

This AIS pattern has been previously reported in three cetaceans with pulmonary edema; two geriatric bottlenose

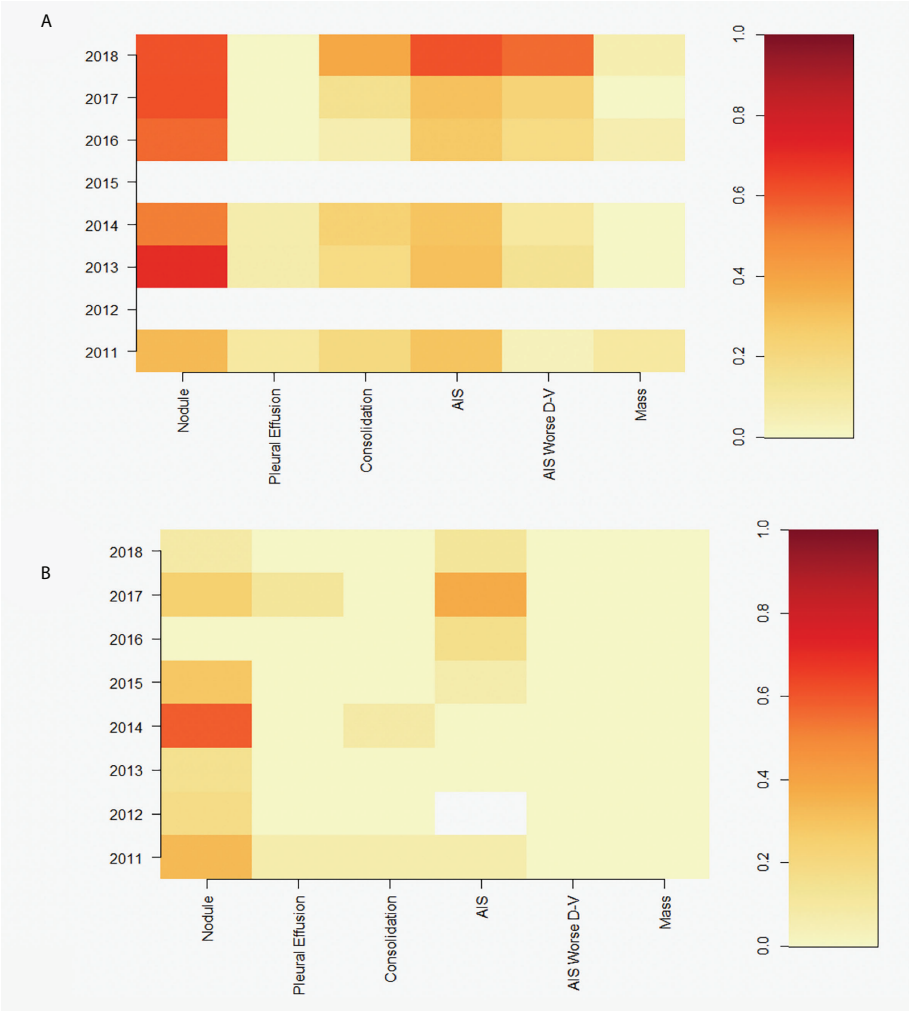

FIGURE 4
Heatmaps showing prevalence of pulmonary nodules ('nodule'), pleural effusion, pulmonary consolidation ('consolidation'), alveolar interstitial syndrome ('AIS'), AIS with increased ventral severity ('AIS Worse D-V'), and pulmonary masses ('mass') diagnosed between 2011-2018 in dolphins that were alive at the time of the DWH spill in **(A)** Barataria Bay (N=132 dolphins) and **(B)** Sarasota Bay comparison site (N=85 dolphins). White cells represent missing data. Colors represent prevalence of conditions, as represented in color key on the right.

dolphins with congestive heart failure (Smith et al., 2012) and one mature vaquita porpoise (*Phocoena sinus*) that developed pulmonary edema secondary to capture myopathy (Rojas-Bracho et al., 2019). However, we considered pulmonary edema unlikely in most BB dolphin cases given the lack of additional clinical findings to support this diagnosis. Although a concurrent cardiac health study showed a higher prevalence of heart chamber abnormalities in BB dolphins than SB dolphins (e.g. thinner left ventricular walls, smaller left atria, tricuspid valve prolapse and thickening, aortic valve thickening) as diagnosed with echocardiography (Linnehan et al., 2021), the cardiac abnormalities in most animals were not considered significant enough to cause clinical manifestations. Additional data would be needed to determine if low-grade underlying cardiac abnormalities could lead to secondary pulmonary edema, specifically under brief capture conditions.

Chronic, progressive pulmonary fibrosis was also considered as a potential explanation for the AIS seen in BB dolphins. Pulmonary fibrosis occurs as a consequence of significant lung injury and is associated with scarring that can progress over time and eventually lead to death. There are numerous types and causes of pulmonary fibrosis, including etiologies related to environmental exposure to chemicals and pathogens (Huang and Tang, 2021; Vlahovich and Sood, 2021; Moss et al., 2022). Although pulmonary fibrosis hasn't been definitely diagnosed with ultrasound in dolphins, ultrasound findings have been described in human patients. These include pleural thickening, pleural irregularity, and an increase in B-line (or ring-down)

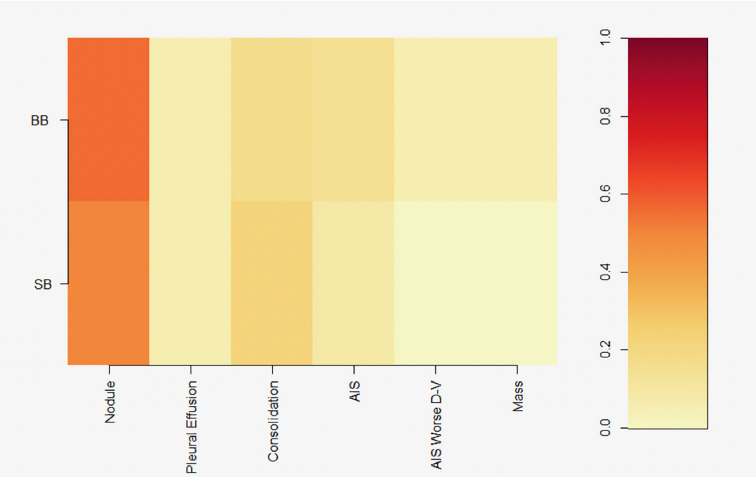

FIGURE 5
Heatmap showing prevalence of pulmonary nodules ('nodule'), pleural effusion, pulmonary consolidation ('consolidation'), alveolar interstitial syndrome ('AIS'), AIS with increased ventral severity ('AIS Worse D-V'), and pulmonary masses ('mass') diagnosed between 2011-2018 in dolphins born after the DWH spill. BB, Barataria Bay; SB, Sarasota Bay. Colors represent prevalence of conditions, as represented in color key on the right.

artifacts that can coalesce with severity to create a 'white out' appearance (Manolescu et al., 2018). Although pleural thickness measurements have not been standardized in dolphins, we subjectively noted thickened and irregular pleura in the majority of BB dolphins, more severe ventrally than dorsally, and dolphins with severe AIS had a characteristic 'white-out' appearance to the lung. Therefore, it is reasonable that at least some of the BB dolphins examined had pulmonary fibrosis. Even so, there is still no clear explanation for why the fibrosis would worsen ventrally, unless it reflected a gravity-dependent inhalation pattern or previous aspiration injury within the lungs that progressed over time.

Pulmonary angiomatosis has been observed in stranded Gulf of Mexico dolphins and can cause interstitial and pleural thickening, often increasing with age (Venn-Watson et al., 2015b). While the potential contribution of underlying pulmonary angiomatosis to AIS needs to be further examined, there are no current data to support that angiomatosis worsens ventrally in the lung, or would be more prevalent in BB versus SB dolphins. A critical need exists to collect and study the carcasses of BB dolphins. Histopathologic analysis of their lung tissue could help determine if chronic progressive pulmonary fibrosis, or another pulmonary interstitial disease, has emerged as a health issue in BB dolphins as a consequence of the DWH oil spill.

Pulmonary damage and subsequent respiratory compromise are not unusual findings several years after exposure to an oil spill. Respiratory symptoms following inhalation exposure from various spills were reported in humans, including the DWH disaster (Alexander et al., 2018; Rusiecki et al., 2018). Experimental studies involving fish and mice exposed to DWH oil and/or associated chemicals reported respiratory injury (Brown-Peterson et al., 2015; Jaligama et al., 2015; Pan et al., 2018). Potential mechanisms of primary injury have been compared across taxa, including oxidative damage, cellular damage, and cellular necrosis (Takeshita et al., 2021). Secondary pathways have also been investigated, including immunotoxicity, cardiotoxicity, and chronic stress.

Based on the comprehensive examination of all available data collected to date, chronic pulmonary disease was likely a significant factor in the overall poor health of dolphins living within the DWH oil spill footprint. There were additional consequences to dolphins that sustained this injury. To help define clinical significance to individual animals, oxygenation and blood gas analyses were conducted (2016 and 2017), which identified evidence of compensatory acid-base disturbances in dolphins with lung disease (Sharp et al., 2017). Poor pulmonary health and acid-base imbalances could contribute to the sustained high rates of dolphin reproductive failure in BB dolphins (Lane et al., 2015; Kellar et al., 2016; Smith et al., 2020), as maternal illness and related adverse health outcomes could put pregnancies at risk and impact a female dolphin's ability to adequately care for her calf. Additionally, overall health scores in BB dolphins showed that dolphin population health has not improved over time (2011-2018) and in some cases has worsened (Schwacke et al., 2022).

A slight increase in prevalence of moderate lung disease was seen in pre-spill SB dolphins (comparison site) sampled during 2016 and 2017. Sample size for this cohort was very limited (5 in 2016; 8 in 2017), so caution is warranted when interpreting these

findings. Furthermore, there were no documented cases of pulmonary masses, pulmonary consolidation, or ventrally severe AIS in SB during 2017 or 2018. Additionally, no cases of severe pulmonary disease were diagnosed in SB during the entire post-DWH disaster study period, compared to a prevalence of 0.23 in 2017 and 0.22 in 2018 in oiled BB.

Dolphins born *after* the DWH oil spill did not have an elevated prevalence of pulmonary disease. In fact, the prevalence of moderate to severe lung disease in BB dolphins born post-spill was similar to SB dolphins not impacted by the DWH oil spill, which is an encouraging finding for this generation of BB dolphins. Although these animals were relatively young at the time of exam (<10 years old), our previous study showed that age was not a significant factor in the prevalence of moderate to severe lung disease in BB dolphins (Smith et al., 2017). Additional monitoring studies will be needed to determine if the prevalence of lung disease in dolphins born post-spill remains similar to SB over time.

This study showed strong evidence of chronic and potentially progressive respiratory injury in bottlenose dolphins living within the DWH oil spill footprint. Other cetacean species living farther off-shore were also exposed, but their health was difficult to evaluate and few data exist to determine the potential adverse health impacts. However, the nearshore bottlenose dolphin data suggests that any cetacean with a similar exposure to DWH oil or its byproducts could sustain a similar injury. Long-term monitoring of dolphin populations living within the oil spill footprint is critical to fully understand the potential for and timeline of individual and population recovery from the impacts of a such a large-scale oil spill event, and to extrapolate impacts on other cetacean populations in the NGoM and elsewhere. As long-lived, air-breathing mammals that live, feed, and breed along the coastline, dolphins can be considered a sentinel species and provide valuable insight into the chronic effects of oil and associated contaminants on animal, human, and ecosystem health.

Data availability statement

Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (doi: 10.7266/N7H41PTV, 10.7266/n7-76aj-rp39, 10.7266/n7-sv57-1h12).

Ethics statement

The animal study was reviewed and approved by National Marine Fisheries Service's Institutional Animal Care and Use Committee and Mote Marine Laboratory's Institutional Animal Care and Use Committee.

Author contributions

CS: conceptualization, methodology, formal analysis, investigation, writing – original draft, visualization, supervision, project administration, funding acquisition. TR: conceptualization, methodology, writing – review and editing, supervision, resources, project administration, funding acquisition. FG: investigation, formal analysis, writing – review and editing, supervision, project administration, funding acquisition. MI: investigation, formal analysis, writing – review and editing, funding acquisition. KC: investigation, formal analysis, writing – review and editing. RT: formal analysis, data curation, validation, writing – review and editing. FT: investigation, writing – review and editing. EZ: investigation, writing – review and editing. JM: data curation, synthesis, and analysis, writing – review and editing. VC: investigation, writing – review and editing. JM: investigation, writing – review and editing. WM: investigation, data synthesis, writing – review and editing. TS: investigation, writing – review and editing. AB: data synthesis, writing – review and editing. RW: investigation, writing – review and editing, resources, funding acquisition. LS: conceptualization, methodology, statistical analysis, validation, writing – review and editing, visualization, project administration, funding acquisition. All authors contributed to the article and approved the submitted version.

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org (doi:10.7266/N7H41PTV, 10.7266/n7-76aj-rp39, 10.7266/n7-sv57-1h12).

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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An insight into gill microbiome of Eastern Mediterranean wild fish by applying next generation sequencing

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Bacterial diseases of marine fish inflict significant economic damage to fisheries and aquaculture and pose an increasing risk to public health. When addressing fish disease, an accumulating body of research suggests adding another factor to the classic epidemiological triangle of host-environment-pathogen: the microbiome. The gills, being a gateway into the fish body and bearing an important role in fish homeostasis, have been found to be a proxy of the gut microbiota as well as reflecting the microbial communities of surrounding water. In this study, 16S rRNA amplicons of bacterial DNA extracted from the gills of 89 asymptomatic specimens of three wild fish species (*Pagrus caeruleostictus*, *Scomber colias* and *Saurida lessepsianus*) were sequenced using Next Generation Sequencing methodology (NGS). Data analyses revealed the presence of 41 potentially pathogenic species, including several zoonotic agents. Five genera known to include widespread and potentially pathogenic species were chosen for further investigation: *Photobacterium, Shewanella, Staphylococcus, Streptococcus* and *Vibrio*. Of these, *Photobacterium* and *Shewanella* proved the most prevalent and abundant, making up 30.2% and 11.3% of the Bluespotted seabream (*P. caeruleostictus*) gill microbiome alone. *Photobacterium damselae* and *Shewanella baltica* were most common at the species level. The remaining genera - *Vibrio, Staphylococcus* and *Streptococcus* – were less prevalent, and at a species level were comprised of only 1–4% potentially pathogenic representatives. Gill microbiomes exhibited host species specificity, with strong correlations between certain bacterial taxonomic groups. No definite obligatory pathogenic bacteria were found in this study, and it was suggested that pathogenic species are present as either covert pathobionts or as opportunists of the fish found to host them.

KEYWORDS

Photobacterium, *Shewanella*, *Staphylococcus*, *Streptococcus*, *Vibrio*, Marine fish, Wild fish pathogens, *Gill microbiome*

Introduction

In many areas of the world, one of the most common horizontal transmission routes of pathogens into wild fish is the rapidly growing mariculture cage-farm industry (Arechavala-Lopez et al., 2013; Barrett et al., 2019; Shea et al., 2020). A reverse pattern of pathogen transmission can also be observed from wild fish to farm stocks (Arechavala-Lopez et al., 2013). Fish cage farms have become ecological hotspots, releasing a steady source of residual uneaten feed and providing a refuge for small-bodied fish species in an otherwise unsheltered open sea habitat. The farms also present an opportunity for replenishment for migratory fish (Shea et al., 2020) and become an attraction for predators (Papastamatiou et al., 2010; Piroddi et al., 2011; Barash et al., 2018). Today, over a quarter of globally farmed fish species are non-native to their rearing environment (Atalah and Sanchez-jerez, 2020), which means in addition to competing with native wild populations over local resources, mariculture escapees pose a risk for introduction of alien pathogens to naïve hosts. The effects of mariculture are coupled with many other anthropogenic factors that increase risk of disease outbreaks in wild fish, including increases in sea surface temperature, pollution and structural alterations to ecosystems through development and industry (Harvell et al., 1999; Halpern et al., 2008; Lejeusne et al., 2010; Nguyen and Liou, 2019). Human-driven changes in the ocean environment directly impact the health of fish. When faced with physiochemical conditions outside of their optimal range, fish may become stressed and immunosuppressed, lowering their defenses against agents of infectious disease (Johnson et al., 1992; Conte, 2004). The epidemiological triangle, describing such interactions between a host, a pathogen, and their environment (King et al., 2019), forms the basis of research aimed at understanding the effects of disease on marine animals (Andrade et al., 2017; Elarabany et al., 2017; Wang et al., 2018; Genin et al., 2020; Zarantoniello et al., 2021).

In recent years, there has been great interest in adding the contribution of the microbiome to this complex interplay, applying the concept of the holobiont, a host with all of its associated microorganisms, to disease research. The intimate partnership between hosts and their symbiotic microbiota plays a significant role in host maintenance and well-being, contributing to metabolism, immune system maturation, and additional defenses against pathogenic invaders (Aschenbrenner et al., 2016; Ramsey et al., 2016; Apprill, 2017; Vorburger and Perlman, 2018). Shifts in this microbial composition due to external pressures from natural or anthropogenic changes in the environment (Halpern et al., 2008; Pérez-Ruzafa et al., 2018; Nguyen and Liou, 2019), or internal physiological pressures (Yildirimer and Brown, 2018), may lead to substantial consequences for the host, and maintaining the balance of the microbiome has been shown to be of importance for maintenance of fish health (Llewellyn et al., 2014). Microbiota composition shifts may serve as early-warning bioindicators, enabling assessment of the host's health even before clinical signs become visible. Combined with data on shifts in pathogen prevalence, these aspects become key factors in understanding the intricacies of host-pathogen relations.

Previous fish microbiome studies focused mostly on skin and intestinal microbiota (Ni et al., 2013; Liu et al., 2016; Egerton et al., 2018; Tarnecki et al., 2019; Krotman et al., 2020), and occasionally on other organs, such as kidneys and liver (Sevellec et al., 2014; Meron et al., 2020). These studies found that gut, kidney microbiota are deeply influenced by fish trophic levels and diversity of their prey, while skin microbiota is both highly adaptive and affected by qualities of the ambient water. Gills, however, are receiving increasing attention, as they may be sampled non-destructively from live fish (Merrifield and Rodiles, 2015; Mohammed and Arias, 2015). In addition to their role in gas and waste exchange (Evans et al., 2005), gills are a gateway into the fish body and an important site of mucosal immunity (Salinas, 2015), constantly in direct interaction with the aquatic environment and its associated microbes. To a certain extent, the gill microbiome reflects the microbial composition of the water, including pathogens present (Kuang et al., 2020). Understanding the mechanisms of pathogen adherence to and entry through the gill mucosal barrier, and the potential impact of the microbiome and surrounding environment on this process, is an ongoing challenge in aquaculture. To date, only a handful of studies aimed at farmed (Brown et al., 2019; Rosado et al., 2019; Minich et al., 2020) or wild fish (Hess et al., 2015; Minich et al., 2020) have been published on the gill microbiome. Pratte et al., (2018) studied reef fish and found that gill and intestinal microbiomes from the same individual showed greater similarity than respective gill or intestinal microbiomes from different individuals, and these authors concluded the presence of a core microbiome amidst the intra and inter-species variances. The gill microbial community, then, could be a representative metric for the total fish microbiome.

Due to its cost-effectiveness and relative accuracy (Caporaso et al., 2011; Vayssier-Taussat et al., 2013; Walters et al., 2015), the

use of 16S rRNA amplicon sequencing is considered common practice in such studies aiming to elucidate the composition of bacterial communities (Sevellec et al., 2014; Mohammed and Arias, 2015; Pratte et al., 2018; Krotman et al., 2020; Meron et al., 2020; Minich et al., 2020). A certain tradeoff exists between accuracy at the species and subspecies level and the ability to comprehensively screen bacterial communities (Ghyselinck et al., 2013; Martínez-porchas et al., 2016). Improvements in primer design and bioinformatics tools have helped bridge that gap, and enabled both higher reliability of NGS screening and the additional benefit of discovering novel species (Al-Hebshi et al., 2015; Johnston et al., 2017; Abu Fanas et al., 2021; Greay et al., 2021). In the present study, we use 16S rRNA NGS screening to provide an analysis of the gill microbiome of three fish species, in order to assess community composition and the presence of potential pathogens. Furthermore, this study aims to show that this method is useful in detecting multiple fish pathogens in parallel and finding correlations between pathogenic species residing together.

Materials and methods

Fish collection

All fish samples used were collected during a trawler survey conducted during May–June 2020, at depths of 20–80m, as part of a biannual survey, conducted by the Israeli Oceanographic and Limnological Research center (IOLR), in Haifa, Israel. The surveys are carried out at constant locations southwest of Ashdod, and eight kilometers away from cultured fish cages. Fish were immediately placed on ice and transferred to the lab. Eighty-nine fish individuals were collected: Atlantic chub mackerel (*Scomber colias*; n = 40), Bluespotted seabream (*Pagrus caeruleostictus*; n = 25) and Lessepsian lizardfish (*Saurida lessepsianus*; n = 24). Some of the samples were dissected fresh while others were frozen at −20°C to be later thawed and necropsied.

Tissue sampling

Frozen specimens were gradually thawed in small batches, weighed and measured, and were then dissected aseptically according to an established fish necropsy protocol (Yanong, 2003). Gills tissue samples were gently removed and placed in predesignated test tubes, then frozen at a temperature of −80°C until undergoing DNA extraction. *P. caeruleostictus* samples ranged in length between 11.4-20.4cm and in weight between 23.4-153.4g. *S. lessepsianus* samples ranged in length between 15.2-30.2cm and in weight between 21.7-230.7g. *S. colias* samples ranged in length between 13.2-19.5cm and in weight between 18.6-71.8g.

DNA extraction

Extractions of DNA were done using the GeneMATRIX Soil DNA Purification Kit (EURx, Gdańsk, Poland), following the manufacturer instructions for tissue lysates, with an additional two hour incubation at 55°C following suspension of the sample tissue in the kit-provided lysis buffer. DNA quality was examined by NanoDrop spectrophotometry analysis and agarose gel-electrophoresis.

PCR amplification and amplicon sequencing

Total DNA extracts were used as template for amplification of partial 16S rRNA gene sequences, at the V4 hypervariable region. Amplicons were generated using a two-stage PCR amplification protocol as described previously (Naqib et al., 2018). Each of the first stage PCR reactions consisted of a total of 50µl in volume and included: 25µl of GoTaq Green Master mix (Promega, Fitchburg, WI, USA), 2µl of mixed forward and reverse primers (in a concentration of 1nM each), 2µl of bovine serum albumin (BSA), 18µl of ultra-purified water (UPW) and 3µl of 80ng/µl template DNA. The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) compatible with Access Array™ primers for Illumina sequencers (Fluidigm, South San Francisco, CA, USA) (Caporaso et al., 2012). The primers used for amplification were (linker sequences in **bold**): CS1_518F: 5' – **ACACTGACGACATGGTTCTACA**CCAGCAGCCGCGG TAATACG – 3' (Nakasaki et al., 2009) and CS2_806Rc: 5' – **TACGGTAGCAGAGACTTGGTCT**GGACTACNVGGG TWTCT – 3' (Walters et al., 2015).

The PCR conditions were as follows: 10 cycles of denaturation at 95°C for 15s, annealing at 60°C for 15s and elongation at 72°C for 30s; followed by 10 cycles of denaturation at 95°C (15s), annealing at 55°C (15s) and elongation at 72°C (30s); continued with 10 more cycles at 95°C (15s)/50°C (15s)/72°C (30s); and then 5 additional cycles with yet another change of annealing temperature, performed at 62°C. The PCR concluded with 2 minutes of incubation at 72°C, before being lowered to 4°C for one hour (or until samples were removed). Amplicons were sent to UIC Sequencing Core (Chicago, IL, USA), in which a second PCR amplification was performed in 10 microliter reactions in 96-well plates using MyTaq HS 2X mastermix (Bioline, Taunton, MA, USA). Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). One microliter of PCR product from the first stage amplification was used as template for the 2nd stage, without cleanup. Cycling conditions were 95°C for 5 minutes,

followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". Libraries were then pooled and sequenced with a 20% phiX spike-in on an Illumina Miniseq sequencer employing a mid-output flow cell (2x150 paired-end reads). Final library preparation, pooling, and sequencing were performed at the Genome Research Core (GRC) at the University of Illinois at Chicago (UIC).

Sequence data processing

Detailed information regarding the sequence data processing is provided in the Supplementary Information File. In brief, sequence data was analyzed using the Dada2 pipeline (Callahan et al., 2016) using R package 'dada2' (version 1.14.1). Error rate estimation was carried out in order to sample nucleotides and reads for model building randomly across all samples. The dada2 algorithm was implemented for error correction and a count table containing the amplicon sequence variants and counts per sample was produced. For each amplicon sequence variant (ASV), taxonomy (up to the species level) was inferred by alignment to the Silva non-redundant small subunit ribosomal RNA database (version 138) using dada2 commands 'assignTaxonomy' and "addSpecies" with minimum bootstrap value set to 80%.

Data analysis

All data filtering parameter settings are detailed in the Supplementary Information File. In short, for data analysis and generation of figures, the online tool MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml) was used (Dhariwal et al., 2017; Chong et al., 2020). Taxonomy labels were assigned using the SILVA taxonomic framework (https://www.arb-silva.de/documentation/silva-taxonomy/). Initial analyses provided 189 bacterial ASVs identified to the taxonomic level of species, with 177 unique values (i.e., species). All 177 species were searched in the literature using their species name separately and together with conjugations of 'Pathogen', 'Infection' or 'Disease', with and without reference to fish/humans. In addition, sequences belonging to "pathogenic" genera were run through BLAST. This enabled identifying four more species and raised the total number of species to 181. Forty-one species were categorized as pathogenic to marine animals and/or humans. A literature-based scale was built applying several categories for their range of pathogenicity: from 'Unknown' to 'Rarely', 'Pathobiont', 'Opportunistic', 'Yes' and 'Obligatory'. Species were labeled 'Unknown' whenever the literature provided no evidence of pathogenicity whatsoever. 'Rarely' is a term used in the literature almost solely in reference to human pathogens. The commonly used term 'Facultative' was split into 'Pathobiont' and 'Opportunistic', differentiating them by defining the former as a mutualistic symbiont becoming virulent under certain conditions, while the latter refers to a commensal symbiont of pathogenic capabilities, a 'hitchhiker' that usually does not provide useful services to the host – nor causes harm – but turns virulent when conditions are favorable of it. 'Yes' marks an uncertainty whether the pathogen should be categorized as 'Rarely', 'Pathobiont' or 'Opportunistic'. 'Obligatory' refers to obligatory pathogens, meaning they always express virulence. This is a rare attribute found in bacteria and no obligatory pathogens were found in this study.

Phylogenetic trees

A detailed account of the parameters used for creating trees is given in the Supplementary Information File. Briefly, sequences identified as belonging to the several genera chosen for deeper enquiry were uploaded to Silva (https://www.arb-silva.de/aligner/) for preparing phylogenetic files (Quast et al., 2013; Yilmaz et al., 2014; Oliver et al., 2017). The ACT (Alignment, Classification and Tree Service) tool was used (SINA v1.2.11) (Pruesse et al., 2012). Output TREE format files were extracted for visualization with the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

All of the 89 fish collected appeared healthy both externally and internally upon inspection and necropsy. The community structure of the gill samples differed between fish species (Figure 1). Atlantic chub mackerel (ACM; *Scomber colias*) exhibited a higher and richer composition (Simpson index average: 0.9) than the Lessepsian lizardfish (LLF; *Saurida lessepsianus*) and Bluespotted seabream (BSSB; *Pagrus caeruleostictus*), which displayed indexes of 0.75 and 0.81, respectively. The LLF had the highest variance between samples. In all three fish species, low-richness outliers did not display an unusual increase in pathogenic agents' presence. A comparison of compositions (Figure 2) shows a clustering of microbiomes among species, with ACM displaying a community structure least similar to the others, and BSSB sharing most of its microbiome with the two other species.

An interaction network (Figure 3), expressing the strength of ties between bacteria genera to each other and their tendency to be hosted by the different fish species, highlights three main cohorts: the '*Psychrobacter* cohort', the '*Photobacterium* cohort' and the '*Staphylococcus-Streptococcus* cohort'. The *Psychrobacter* cohort was the most diverse and contained few genera associated with pathogenicity. It was mostly associated with ACM. The '*Photobacterium* cohort' expressed correlation especially to BSSB

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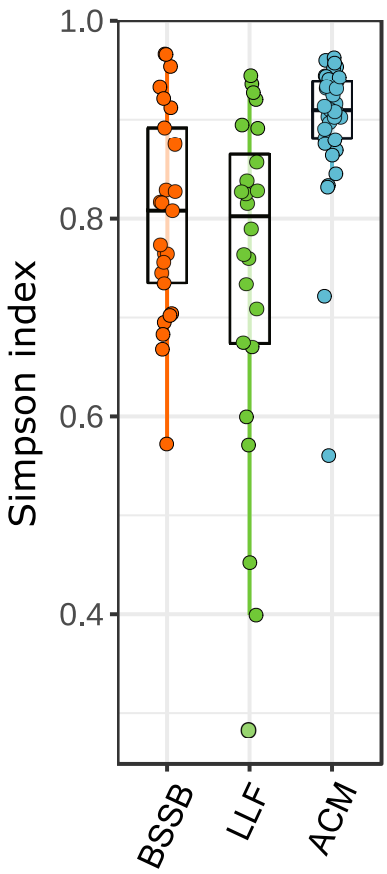

FIGURE 1
Community structure of fish gills samples. Data clustered by fish species. Kruskal-Wallis statistic: 24.928, $P<0.001$.

samples, and presented ties between the Gram-negative Gammaproteobacteria class members *Vibrio*, *Aliivibrio*, *Photobacterium* and *Shewanella*, together with *Cetobacterium* (class Fusobacteriia). The *'Staphylococcus-Streptococcus* cohort' was most strongly associated with LLF, and exhibited correlations between Gram-positive bacteria *Staphylococcus*, *Streptococcus* and *Gemella* (Bacilli) with *Actinomyces*, *Cutibacterium*, *Micrococcus* and *Rothia* (Actinobacteria). It also exhibited correlations with the Gram-negative *Cloacibacteria* (Bacteroidia) and *Enhydrobacter* (Gammaproteobacteria).

The relative abundance of each genus by fish type further supports species-specific microbial composition (Figure S2). The ACM gill microbiome was predominated by *Psychrobacter* (28.3%), and BSSB by *Photobacterium* (30.2%). These two genera were notably present in all three species, alongside *Shewanella*. The *Cetobacterium, Aliivibrio, Cutibacterium, Vibrio, Rothia, Staphylococcus* and *Streptococcus* genera were distributed in lower abundance between two or three species. Over 65% of the LLF, 27% of the ACM and 18% of the BSSB microbiome reads were classified as "Not-assigned" with an additional set of reads in each returned as "others", a summary of low count genera. The genera known to include many potential pathogenic species – *Photobacterium, Shewanella, Staphylococcus, Streptococcus*, and *Vibrio* – were also unevenly distributed between fish types (Figure 4). From this figure there are several observations to be made: (i) except for *Staphylococcus*, BSSB is host to a larger percentage of potentially pathogenic bacterial species than the other two fish species; (ii) error bars across all three fish species indicate that large variances occur amongst samples from each fish species, especially in the top quarter percentile of samples.

The NGS data analyses resulted in an output of 5,798 unique amplicon sequence variants (ASVs) of which 5,717 were identified as bacteria, 15 as archaea, five as eukaryotes and the rest unidentified. None of the ASVs appeared in 100% of the samples, nor in 100% of the samples of any specific fish species. Of those bacterial ASVs, 189 were initially identified to the taxonomic level of species, with 177 unique values (i.e., species). Of this list, 41 species were identified as bearing some pathogenic potential to humans and/or marine animals (Table S1): 36 had varying human clinical relevance or zoonotic potential. These were divided into the following taxonomic classes: Gammaproteobacteria (n = 15); Actinobacteria (n = 10); Bacilli (n = 8); Campylobacteria (n = 1); Alphaproteobacteria (n = 1); and Fusobacteriia (n = 1). The literature also revealed that of the 41 potentially pathogenic species, a total of 14 were known marine animal pathogens (meaning, some of those potentially pathogenic to humans may also cause disease in marine wildlife). These 14 species were of the taxonomic classes Gammaproteobacteria (n = 9); Bacilli (n = 3); and Bacteroidia (n = 2). Thirteen of the fish pathogens appeared in samples belonging to ACM, six in LLF and four in BSSB. The results are visualized in Figure 5. It shows *S. baltica* to be highly prevalent in these fish species (found in 95% of ACM, 46% of LLF and 92% of BSSB), and that *P. damselae* was also prevalent (73%, 38% and 84% of the ACM, LLF and BSSB samples, respectively). In contrast, pathogenic *Streptococcus*, *Staphylococcus* and *Vibrio* species were found less frequently.

Phylogenetic analysis of these five genera of interest demonstrated the most closely related reference species to the ASVs with pathogenic potential. The *P. damselae* clade (Figure S4) appears divided between ASVs associated with *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*, in which the former is predominant – both in number of ASVs and total number of reads. In total, *P. damselae* makes up >30% of the genus' ASV reads. The *Shewanella* phylogenetic tree (Figure S5) is comprised of numerous ASVs, as this genus is the most prevalent (though not most abundant) of all genera analyzed. The *S. baltica* associated ASVs were responsible for >70% of all *Shewanella* reads. The trees of *Staphylococcus*, *Streptococcus* and *Vibrio* (Figures S6–S8, respectively) are similar in terms of the pathogenic/non-pathogenic ratios they exhibit: between 1–4%. This data is summarized in Figure 6.

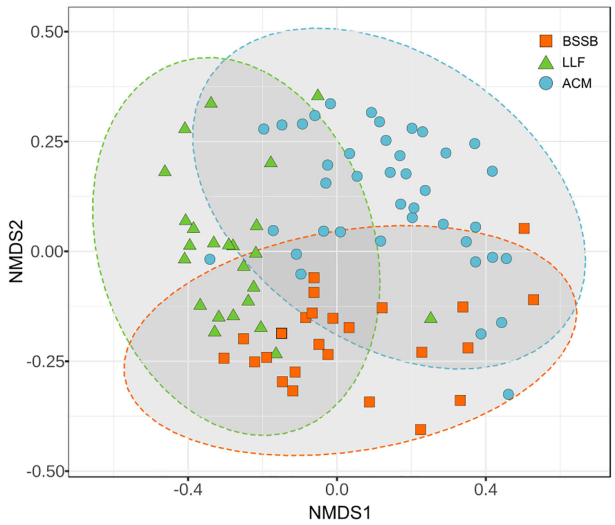

FIGURE 2
Comparison of compositions of the gills' microbiome between the sampled fish species. PERMANOVA: F: 7.7605, R^2: 0.1529, P<0.001, NMDS stress = 0.1740.

Discussion

The use of fish gill microbiomes to assess pathogen prevalence is gaining importance and becoming increasingly routine (Hess et al., 2015; Pratte et al., 2018; Brown et al., 2019; Rosado et al., 2019; Minich et al., 2020). However, to the best of our knowledge, this is the first study to be carried out in the Eastern Mediterranean. In previous studies, the presence of pathogens was inferred from taxonomic levels higher than species (Pratte et al., 2018; Rosado et al., 2019), sometimes supported by data on shifts in microbiome composition between treatment and control groups (Hess et al., 2015; Mohammed and Arias, 2015; Brown et al., 2019; Minich et al., 2020) or spatio-temporal differences (Minich et al., 2020). In the current study, we based our findings regarding pathogens only on ASVs that we could identify to the species level at a high degree of certainty. The data show that Atlantic chub mackerel (ACM), which was found to host the richest microbial community of the three fish species, also shows the largest total number of bacterial species with pathogenic potential. The microbiome of ACM included 35 out of the total of 41 pathogenic bacterial species found (and 13 of the 14 fish pathogens), while Lessepsian lizardfish (LLF) had 26 (6/14) and Bluespotted seabream (BSSB) just 6 (5/14 fish pathogens). A possible explanation for this result may be the pelagic-migratory nature of ACM, which means that it passes through diverse geographic zones, where it may accumulate a variety of bacteria on its gills. BSSB has a relatively high abundance of *Photobacterium*, and within the *Photobacterium* genus, ~30% of the reads belong to a specific and well-known pathogenic species. This entails that ~10% of BSSB gill microbiome is *P. damselae*. Had this putative pathogen been obligatory, many of the samples would have shown signs of infection. Since this was not the case, it raises the question whether the two subspecies of *P. damselae* (a multi-gene PCR array for sub-speciation was not performed in this study) are opportunistic or pathobionts. Previous studies suggest that a genetic diversity within *P. damselae* subsp. *damselae* strains means disease outbreaks in fish are most likely caused by multiclonal populations, containing several complementing virulence factors (Terceti et al., 2016). Cases in which *P. damselae* is highly prevalent in fish without causing disease may reflect the need for a few variants of this pathogen to 'join forces' to create effective infection (Terceti et al., 2016). This is supported by a study conducted on *P. damselae* subsp. *piscicida*, which found variability of virulence in different strains, governed by a protein exotoxin, AIP56 which is secreted by virulent *P. damselae* subsp. *piscicida* in large quantities but not by avirulent strains. This is a key factor responsible for apoptogenic activity targeting fish macrophages and neutrophils (do Vale et al., 2005).

The observed high correlations between Gammaproteobacteria of the *Photobacterium* cohort, and especially the Vibrionaceae members, *Vibrio*, *Aliivibrio* and *Photobacterium*, suggest that the ecological niche existing in the form of BSSB gills provides these genera with preferable conditions. A similar assumption can be made regarding the *Streptococcus-Staphylococcus* cohort and the gills of LLF. In reference to potentially pathogenic species, within the *Streptococcus-Staphylococcus* genera-related sequences, pathogenic species make up only a small percentage of the total reads, and these two genera are comparatively less dominant in terms of relative abundance. On the other hand, *S. baltica*, a bacterium that is not known to be pathogenic to marine animals,

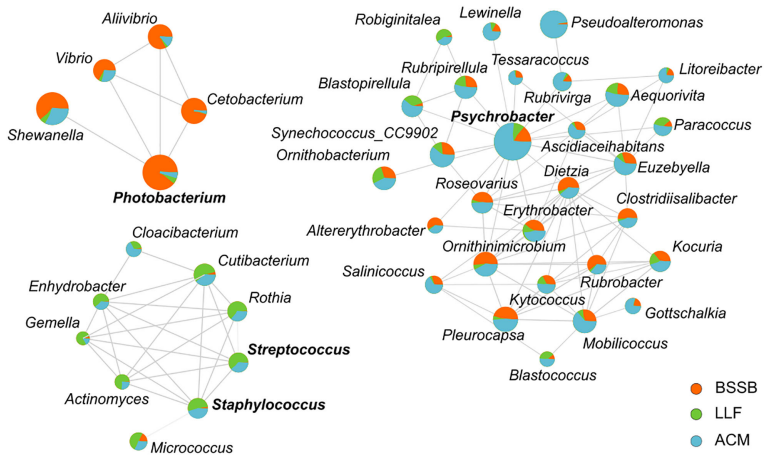

FIGURE 3
An interaction network between bacterial genera, in reference to fish species. Network calculation used Spearman's rank correlation coefficient and the threshold settings placed at: correlation > 0.5; $P<0.05$. Size of circles represents abundance and the coloring associates bacteria with host in regards to mean abundance. Names of 4 genera are emphasized by bold type, in order to identify them as those after which each cohort was named (i.e., *Photobacterium* cohort, *Psychrobacter* cohort and *Staphylococcus-Streptococcus* cohort).

but is rather a major cause of food spoilage (Zhang et al., 2020), dominates *Shewanella*-associated ASVs. A comparison of the pathogenic/non-pathogenic ratios per genus (Figure 1) summarizes these differences. According to our analyses, *Vibrio*, a genus found relatively much less abundant than *Photobacterium*, also comprised almost entirely of non-pathogenic species.

Nevertheless, it is important to remember that separating *Vibrionaceae* members by means of their 16S genes is problematic (Machado and Gram, 2015), therefore better identification may require lengthening the amplicon sequences by using a different set of primers (Morales and Holben, 2009; Martínez-porchas et al., 2016), or by complementing the identification using Multilocus Sequence Analysis (MLSA) – targeting protein-encoding genes such as *toxR* and *rpoD* (Pascual et al., 2010).

While using rRNA amplicon sequencing for the study of microbiomes has great advantages and is extremely useful, its limitations must be considered. In a highly diverse microbiome, different bacteria can display functional redundancy. Hence, a 'function over phylogeny' approach studying the functional characteristics of the microbial community – in addition to the species composition – may be advantageous and more informative (Moya and Ferrer, 2016; Gibbons, 2017; Louca et al., 2017). It was suggested by de Bruijn et al. (2018) (Louca et al., 2017) that the functional characteristics of greatest importance would be the protection of the fish against pathogens, and that this could be achieved by research of gene clusters involved in biosynthesis and production of proteins and metabolites, and studying these genes' frequency, diversity and transcription (Lynn and De Leenheer, 2019). Also, parallel investigation of both phylogeny and function is likely to help cut to a minimum the over- or underrepresentation of certain bacterial families, due to bias resulting from DNA extraction methods (Kashinskaya et al., 2017) and PCR, when relying solely on rRNA amplicon sequencing (Chakravorty et al., 2007; Sinclair et al., 2015; Stoddard et al., 2015).

The formation and function of the gill microbiome can be better understood when considering the much more investigated gut microbiome. During evolution, as the fish body structure developed and became increasingly complex, the adapting mucosa epithelia would have likely required additional resources, higher metabolic rates and improved metabolic capacities. In the ancient ocean, where microorganisms presumably flourished, interactions with fish inevitably formed the basis for creating early microbiomes (Gomez et al., 2013). According to Maynard et al. (2012), in the intestine and microbiome relationship, one counterpart receives a protected gut environment while the other benefits from the microbial highly adaptive metabolic engine that provides essential factors (e.g., vitamins) as well as an improved host ability to obtain nutrients from food. Gill et al., (2006) suggested that the human metabolism is the result of an amalgamation of microbial and human attributes. The microbiome can therefore be viewed as representing an evolutionary collection of species which is integral to the host's constant strive to augment the utilization of its food components (Maynard et al., 2012). In line with this hypothesis, i.e., the acceptance of commensal presence on the mucosal barriers, likely evolved in early vertebrates (e.g., fish), to profit from the ready-made microbial genetic material rather than independently, creating novel metabolic capabilities. It follows, that the greater the microbiome diversity, the better the chances of improving performance. The gill mucosa, as a

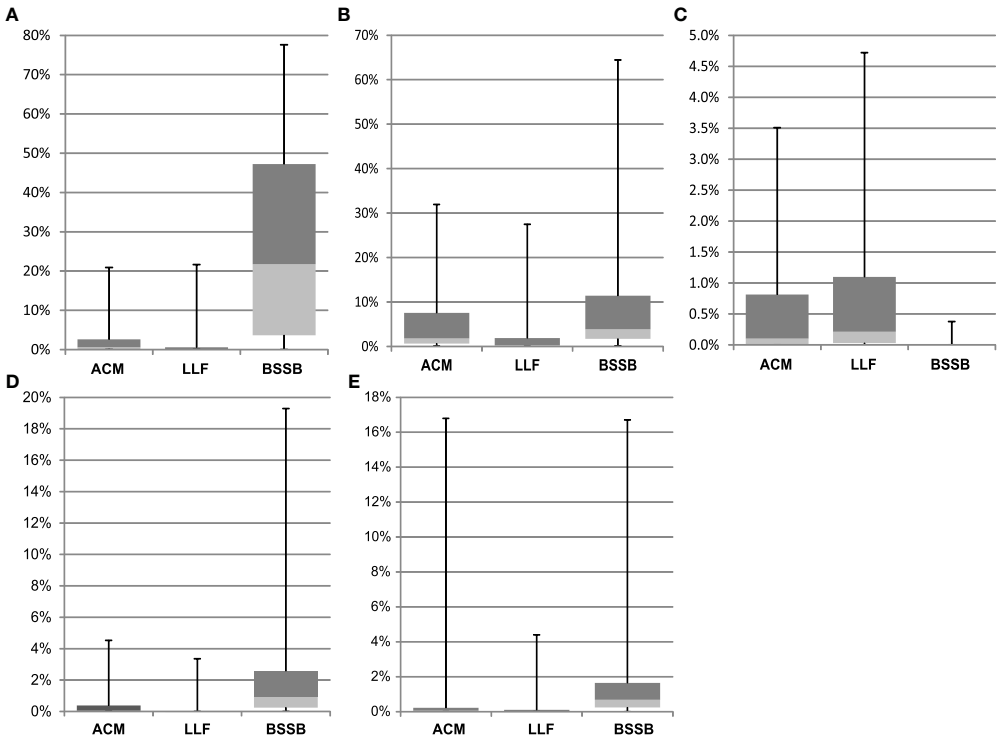

FIGURE 4
Relative abundance of five genera, clustered by fish species. Genera represented: *Photobacterium* **(A)**, *Shewanella* **(B)**, *Staphylococcus* **(C)**, *Streptococcus* **(D)**, and *Vibrio* **(E)**. Error bars depict quarterly percentiles, i.e., 25%, median, 75%, 100%.

semipermeable barrier between the fish and external milieu, would be faced with similar challenges (Koppang et al., 2015).

In the gills, similarly to the gut, the fish immune system must be able to identify commensal bacteria to avoid unnecessary inflammatory reactions triggered when pathogens threaten the intactness of the mucosal barrier (Gomez et al., 2013). The development of such immune tolerance to bacteria has been demonstrated in carp, where repeated anal administration of allogeneic cells has led to loss of allospecific cell-mediated immune response (Sato et al., 2005). Certainly, "false-alarm" inflammatory responses to innocuous bacteria, e.g., resulting in hyperplasia of the branchial epithelium, would not only place an unnecessary burden on the fish vital resources but also compromise the gas exchange processes of the respiratory epithelium.

Apart from host-pathogen relations, bacterial social interactions are another factor affecting virulence expression – both in terms of creating the conditions favoring virulence, and also in the ability to control virulence expression. Bacterial populations experience pressures of conflict and cooperation, which become a major factor in the organization and function of microbial communities (Asfahl and Schuster, 2017). Such interactions are found to be a stabilizing element widespread in *Vibrio* species, creating a protocooperation-based community, which shows increasing growth yield, while creating incentives to prevent a "Tragedy of the Commons" (Bruger and Waters, 2018). It was further shown that cooperative communities include members responsible for "policing", so that when challenged by cheaters, cooperative behavior can persist, provided that four conditions hold: (i) toxin-producers are present; (ii) the cost of toxin production surpasses that of public good production, meaning, policing becomes more expensive than cooperation; (iii) the toxin's harmful effects on the cooperator has to be sufficiently high – in order to counterweigh that policing is more costly than cooperation; and finally, (iv) the toxin's effects on the cheater must be even higher (Lynn and De Leenheer, 2019). An example coinciding with this theory is the pathogenic habits of *Vibrio harveyi*. In one study (Montánchez et al., 2019), this species was found to express virulent genes under heat stress, while cells of its community as a whole suffered extensive fatalities, demonstrating that disease outbreaks due to elevated sea surface temperatures, is but an escape route taken to avoid mortality. This means that pathogenicity in this species is not a display of offensive behavior, but rather a defensive mechanism. Some pathogens can survive for substantial periods in the open water, which explains how fish-cage originated secretions may travel many kilometers with the currents and still affect wildlife (or other cage farm stocks) downstream (Viau et al., 2011; Shapiro et al., 2013). Yet, pathogens are mostly prevalent in hotspots (e.g.,

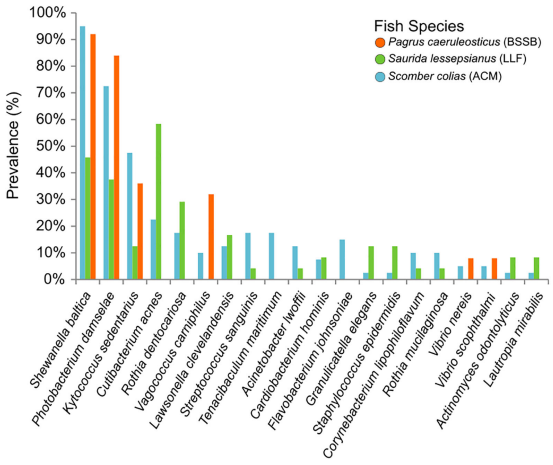

FIGURE 5
Prevalence of the 20 most commonly found pathogens in the gills of the fish sampled. The prevalence (y axis) refers to amount of samples per species found to contain these specific potential pathogens. Pathogens arranged (on the x axis) from the most (overall) prevalent (left) to the least (right).

immunosuppressed animals, naïve hosts, or those associated with anthropogenic pollution-hit areas) (Lyons et al., 2010; Lobelle and Cunliffe, 2011). Therefore, perhaps *Pathogenicity*, being defined as "the quality of producing or the ability to produce pathologic changes or disease" (Shapiro-Ilan et al., 2005), is inherently a trait 'designed' to affect a host. Being host-oriented, the presence of the 'right' host cells is an essential (though not sufficient) prerequisite for expressing pathogenicity. Yet, variances in genotypic attributes between different fish species (even those sharing diets, trophic levels and habitats), will create different 'environmental' conditions within gills (and other fish organs, for that matter), meaning pathogens in these organs will face different microbial communities (Pratte et al., 2018), which in turn may have an effect on the expression of pathogenicity of a given pathogen. Such a mechanism may help explain why asserting whether some less known bacteria are pathogenic (and to which degree), may prove tricky: some pathogens are not necessarily the cause of a certain disease, but are rather secondary agents of it, helping progress it, or just gaining benefit from the change in conditions within the infected organ (Brink et al., 2019). It is known (Laanto et al., 2012) that some pathogens may develop into symbionts with opportunistic pathogenic capabilities, using this ability as a survival tool, which may hint why co-infection is common – an arrival of a non-symbiotic pathogen (due to injury), may cause the host to become immunosuppressed and create conditions favorable of pathogenesis for the symbiotic pathogen.

As a general rule, bacteria of all phyla are known to have certain 'preferable' physio-biochemical conditions in which they thrive, and a range of conditions that can be regarded as 'tolerable'. The Eastern Mediterranean water column displays stratification, with different physiochemical properties existing in different layers that affect the local microbial communities (Techtmann et al., 2015). It was also shown that Gorgonians (Octocorallia, Anthozoa, Cnidaria), which are at the heart of extremely biodiverse ecosystems second only to tropical coral reefs, exhibit great differences in their microbiomes relative to their surrounding waters (van de Water et al., 2017). It is therefore obvious that fish gills offer a niche with a unique set of conditions, inducing the formation of certain microbial communities that are different than those in the water the fishes swim in. However, pathogenesis expressed in fish gills may also be affected by: (i) changes in the gills' microbiome throughout the life-time of the fish (Nagelkerken and van der Velde, 2002; Wilson et al., 2010; Mercier et al., 2012); and (ii) the possible presence of protistan parasites (e.g., ciliates, flagellates, etc.), which frequently facilitate the establishment of secondary microbial species (Gibbons, 2017), as well as many other pathogenic agents – viruses, fungi (Bui et al., 2019), macro and micro-parasites such as helminths and myxosporea (Molnár, 2002; Liyanage et al., 2003; Nguyen et al., 2021).

Fish gills harbor species-specific microbiomes, exhibiting strong correlations between certain taxonomic groups. In the present study, we demonstrated some overlap between the three host species sampled, perhaps expressing a form of core microbiome. The genera which were the focus of the study, *Vibrio*, *Photobacterium*, *Shewanella*, *Staphylococcus* and *Streptococcus* are important members within these fish species gills microbiomes, and at least in the case of Bluespotted seabream, a substantial percent of its gills microbiome is populated by generalist pathogenic species, which are notorious marine pathogens. In conclusion, given that all fish sampled appeared healthy, and based on the notion that pathogenicity is

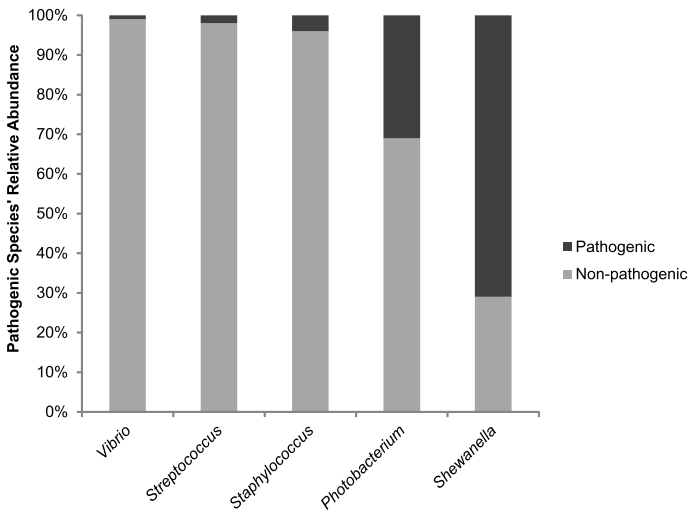

FIGURE 6
A comparison of the Pathogenic/Non-pathogenic ratios per genera of interest. Values represent relative abundance of potential pathogenic species identified out of all ASVs associated with a certain genus.

also influenced by environmental pressure against virulence (coming from microbial community interactions, carrying a strong preference for cooperation over cheating strategies), it can be inferred that pathogenesis is but one of many tools for survival and reproduction that bacteria are equipped with. This in turn explains the fact that pathogens are very rarely obligatory. What it also means is that healthy wildlife populations are not necessarily devoid of pathogens, but have a mix they coevolved with and which protect them from invasions of novel types.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and link to the data can be found below: DRYAD; https://doi.org/10.5061/dryad.wh70rxwr3.

Ethics statement

Ethical review and approval was not required for the animal study because the subjects of research were dead fish provided by another institute conducting an approved ecological study.

Author contributions

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

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1008103/full#supplementary-material

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Lobomycosis-like disease epidemiology, pathology and social affiliations in bottlenose dolphins from Southwestern Gulf of Mexico

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Mycotic diseases are considered a worldwide growing concern related to public health. Lobomycosis like disease (LLD) (*Lacazia loboi*) is a chronic and progressive infection in skin of humans and small cetaceans present in both sides of the Americas, including Mexico but information is still limited. Marine predators are indicators of potential risks for human and wildlife health, including fungal diseases like LLD in bottlenose dolphins. Here we report the first findings of an initial assessment in LLD epidemiology, pathology, and behavioral constraints of coastal bottlenose dolphins (*Tursiops truncatus*) from the Southwestern Gulf of Mexico (SWGM). Overall, LLD prevalence in the population was low, within ranges reported for the species, and only in highly associated unisexual pairs near the Alvarado coastal waters. Photo-identified individuals exhibited an annual increase in average progression for LLD skin lesions. Gross lesions and skin biopsy evidenced mycotic structures and subcutaneous alterations associated to LLD. Habitat quality, demographic, and social characteristics of bottlenose dolphins are likely influencing LLD geographical expansion and temporal prevalence, but global and local climate variability may influence LLD epidemiology, implying a potential risk for human and dolphin health from coastal communities at the SWGM.

KEYWORDS

zoonosis, infectious diseases, marine mammals, wildlife, mycosis, cetaceans, public health

Introduction

Lacaziosis like disease (LLD), formerly known as Lobomycosis, is a mycotic (*Lacazia loboi*) chronic skin disease that affects several species of small cetaceans with potential transmission to humans (Reif et al., 2013). Therefore, coastal bottlenose dolphins (*Tursiops truncatus*) are useful indicators of risk of emerging diseases in human populations (Bossart, 2007, Bossart et al., 2019). Gross findings in the skin of *T. truncatus* affected by LLD are consistent with white to gray nodules that may ulcerate and form large plaques, particularly in fins, head, fluke, and caudal peduncle (Reif et al., 2006; Van Bressem et al., 2007; Murdoch et al., 2008; Ueda et al., 2013). The impact of LLD in marine mammals is unclear, nonetheless, there is a growing concern about its persistence and progression as well as high prevalence, especially in *T. truncatus* populations (Van Bressem et al., 2007; Van Bressem et al., 2015; Siciliano et al., 2008; Daura-Jorge and Simões-Lopes, 2011; Bessesen et al., 2014; Ramos et al., 2018). Immunodeficiency seems to facilitate its occurrence acting as an opportunistic infection (Reif et al., 2009) and as potential death cause due to an eventual immunologic dysfunction in individuals (Bossart et al., 2019). Although, the etiology and epidemiology of LLD in most cetaceans worldwide are still largely unknown and recently associated with *Paracoccidioides brasiliensis* var. *ceti* in *T. truncatus* (Vilela et al., 2016), LLD transmission among individuals has been linked to social behavior in coastal *T. truncatus*, suggesting horizontal contagion and geographic dissemination related to sex (Tenorio-Osorio, 2015; Félix et al., 2019).

Additionally, chemical (e.g., pollutants) and biological (e.g., pathogens) characteristics of marine habitats are thought to play a role in LLD presence and prevalence, particularly in small cetaceans inhabiting sites impacted by anthropogenic activities like inshore or estuarine populations (Bessesen et al., 2014; Rotstein et al., 2009; Van Bressem et al., 2009ab; Félix et al., 2019). However, LLD has also been identified in offshore *T. truncatus*, suggesting an increase in its geographical range (Rotstein et al., 2009). This is relevant considering that mycotic diseases are a worldwide growing public health concern in the face of climate change (e.g., ocean warming) (Seyedmousavi et al., 2015; Gnat et al., 2021). For instance, the expansion of marine mammal foraging areas due to prey shortage during marine warm conditions may increase individual exposure to new and polluted environments (chemical and biological), including pathogens like *L. loboi* (Learmonth et al., 2006; Van Bressem et al., 2009b),

Currently, LLD has been recorded in *T. truncatus* from both sides of the Americas; in the Pacific (Van Bressem et al., 2015, Ramos et al., 2018) and Atlantic (Vilela et al., 2016), including South Africa (Van Bressem et al., 2015), but apparently there are no records of LLD from the Southwestern Gulf of Mexico (SWGM). Therefore, this research aims to provide an epidemiological baseline of LLD in *T. truncatus* from SWGM, including the progression of gross skin lesions in the dorsal fin and their relation to the social behavior of infected individuals, to fill in the geographic gap and improve our overall understanding of LLD presence in small odontocetes.

Materials and methods

Coastal *T. truncatus* from Veracruz state (SWGM portion) have been studied intermittently since the early 1990's using primarily photo-identification (Heckel, 1992; Martínez-Serrano et al., 2011; Morteo et al., 2014, Morteo et al., 2017, Morteo et al., 2019; Bolaños-Jiménez et al., 2021; Bolaños-Jiménez et al., 2022). The studied area comprises roughly three sites in the coastal strip from shore to the 30 – 40 m isobath along 230 km off the waters of 1) Nautla, 2) Veracruz Reef System (VRS), and 3) Alvarado lagoon system (ALV) (Figure 1). Weather variability follows a tropical pattern with three periods: rainy (July-October), windy (November-February), and dry season (March-June) (Bolaños-Jiménez et al., 2022) that provide a variable influx of organic material discharged from the continent to the sea that, in addition to tidal exchange, produce important differences in salinity and temperature (Morán-Silva et al., 2005). Rivers and lagoons sustain local patches of mangrove habitats, where marine sediments and water contain traces of organochlorine pesticides that affect water quality and are toxic to marine species (Vázquez-Botello et al., 2019). Also, artisanal riverine fishing is one of the most important commercial activities carried out all year in the area and antagonistic interactions with *T. truncatus* are frequently recorded (Rechimont et al., 2018; Morales-Rincon et al., 2019).

T. truncatus from the SWGM were photo-identified using their dorsal fins during different survey campaigns at the three sites, during 2002 – 2003 (Nautla and ALV) (photographic negatives), over 2005 – 2007 (digital pictures at the VRS), and 2006 – 2010 (digital pictures at ALV). Individuals were identified by their natural marks according to standard photo-identification procedures using SLR cameras equipped with 70 – 300 mm lenses (Morteo et al., 2014; Bolaños-Jiménez et al., 2022). Only long-lasting marks such as cuts, nicks, and deformities were used for individual identification. All photographs were graded for technical quality (e.g., focus, sharpness, lighting, angle, and proportion of dorsal fin exposure) and distinctiveness. We followed a previously tested protocol for the selection of photographs (Urian et al., 2015), and selected only good-quality images (Q1 and Q2) of individuals with conspicuous permanent markings (D1 and D2) to minimize misidentification. Catalogs were kept and analyzed at the Marine Mammal Laboratory (LabMMar, IIB-ICIMAP) at Universidad Veracruzana (UV). Population estimates for *T. truncatus* of the studied area have yielded between 69 and 636 individuals, with daily abundances of 187 (±132 SD) dolphins for Nautla, over 45 (±23 SD) for the VRS

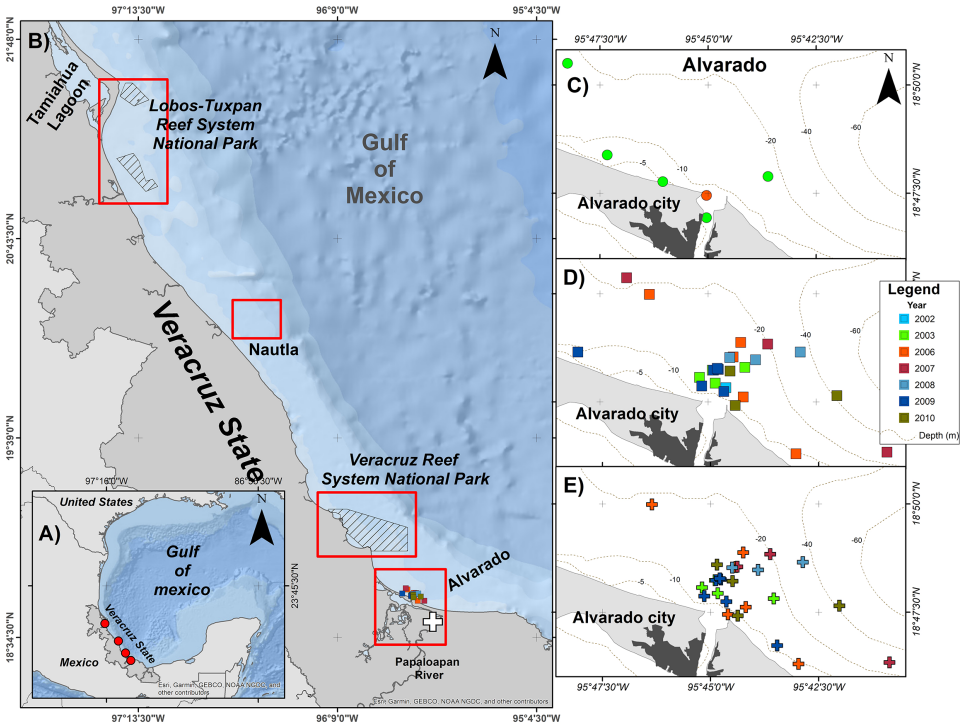

FIGURE 1
Study area along the coastal waters off Veracruz. **(A)** General location of bottlenose dolphin study sites in the Southwestern Gulf of Mexico (red circles). **(B)** Location of the sites with photo-ID catalogues and studies on skin lesions in bottlenose dolphins. **(C)** Recorded sightings of individual EN05. **(D)** individual EN10. **(E)** individual TN15. The white cross shows the location of the stranding of the dead male EN10 in the Papaloapan river.

(Morteo et al., 2019), and monthly abundances between 70 (±14 SD) (Bolaños-Jiménez et al., 2022) and 125 (±52 SD) (Morteo et al., 2017) for ALV, when considering the more resident fraction of the population and the whole sample (residents and transients), respectively.

The presence of LLD was visually analyzed when gross lesions were observed (i.e., raised light grey whitish to pinkish granulomas, nodules, and ulcers) (Van Bressem et al., 2007; Daura-Jorge and Simões-Lopes, 2011). Relative size and progression of LLD were determined by measuring the lesion areas and dividing them by the total surface of the dorsal fin (Daura-Jorge and Simões-Lopes, 2011). Thus, successive images of each individual were projected onto millimeter sheets and the areas were measured. The first and last images of each animal were used to determine changes in the proportion of the affected area. We computed the prevalence (P) of the LLD lesions as the proportion of the animals in the population that have potentially acquired this disease, by dividing the number of affected animals (N_I), by the total number of photo-identified individuals (F_I) and multiplied by 100.

Also, whenever individuals with LLD were identified, photographic records of these animals were used to measure the level of association between them and the other individuals identified within each study area, using SocProg 2.4 (Whitehead, 2009). We used a half-weight index which calculates coefficients of association (COA) between pairs of animals within each social network, considering the total number of times each dolphin was observed and the number of times each pair of dolphins were observed together (Cairns and Schwager, 1987). Values range from zero for a pair of animals that were never seen together to one for animals that are always seen together. The mean association rate for all identified individuals observed on more than 5 opportunities/encounters (Morteo et al., 2014) was used to determine the number of significant pairs by a randomization test (Bejder et al., 1998) using 1 000 iterations. The mean values of non-null associations (i.e., >0) for all animals were calculated and compared against those exclusively from individuals with LLD through a test for differences in means (Tenorio-Osorio, 2015; Félix et al., 2019). All significance levels were set at α= 0.05.

Finally, in February 2022 a dead old (>20 y) (based on photo-ID records and teeth attrition) male *T. truncatus* was found at the Papaloapan River in Tlacotalpan, Veracruz (Figure 1), which exhibited diffuse dermatological gross lesions presumptively linked to LLD and matched one of the adult dolphins in our photo-ID catalog since 2002. Thus, a skin biopsy was collected and preserved in 10% neutral buffered formalin for histology description with hematoxylin and eosin stain. Fungal

identification with Methenamine Silver stain was carried out by the Diagnostic Laboratory at Centro Veterinario de Xalapa, A.C. in Xalapa, Veracruz, Mexico.

Results

Individual photo-identification and LLD prevalence

We analyzed 20, 665 images (Nautla=2,779, VRS=8,664, ALV=9, 212) from 524 individuals at SWGM, only 283 images from three dolphins (EN10, EN05, and TN10) exhibited gross lesions in the dorsal fins presumably related to LLD (Table 1). These individuals were initially classified as males based on their behavioral cues (i.e., synchronized swimming and no affiliation with calves) and later confirmed through visual inspection of their genital area on the field. Thus, the prevalence for all identified dolphins at SWGM was estimated between 0.47 and 0.57% considering the estimated total abundance for the area (i.e., 636 dolphins) and the total of photo-identified individuals (i.e., 524 individuals) (Table 1). However, considering that only individuals within the ALV area were affected with LLD (Figures 2A, B), thus according to photo identified individuals the prevalence ranged from 4.6% (from photo-identified dolphins in 2002 – 2003) to 1.4% (identified dolphins in 2006 – 2010) (Table 1), and between 2.4 and 4.3% when considering the total abundances at Alvarado area (Min-Max: 70 – 125 individuals, see *Materials And methods*).

Temporal progression of LLD gross skin lesions

Early gross lesions described in dorsal fins of the three male *T. truncatus* (EN10, EN05, and TN10) presumably affected by LLD were whitish and small nodular areas in dorsal fins and dorsum (100%; 2003 – 2007) that later raised in middle plaques of verrucous lesions and to light grey whitish and pinkish granulomas (66.6%; 2009 – 2010) in dorsal fins, including edges, anterior dorsum and middle flanks (Figures 2B, C) that finally progressed into whitish extensive plaques of serpiginous aspect, ulcerated with deformation of dorsal fin and presence of raised firm nodules at the beak, head, anterior dorsum, flanks and fluke (33.3%; 2019 – 2022) (Figures 2B–E). Area measurements of the same images from each of the three individuals were repeated three times with an average variation of only 0.25% (± 0.037 SD); also, the measurement error (average coefficient of variation) of different images of each individual within the same sighting was 4% (± 4.1 SD). LLD skin gross lesions in dorsal fins persisted and progressed during the study in all three individuals with an increasing average annual rate estimated at 0.61 (± 0.61 SD) times the size of the original lesion (Table 2). The highest progression rate occurred in individual TN15, followed by EN10 and EN05 photo-identified during 2003-2010 (Figures 2A–E).

Interestingly, the stranded dolphin found in the Papaloapan River in 2022, was recorded 24.5 km upstream (in freshwater) and identified as individual EN10 from the ALV population since 2002, and re-sighted alive in the ALV area in 2019. Total length was 276 cm and body weight was estimated between 280 – 300 kg. The cause of death was undetermined, but the aged male exhibited poor body condition and severe LLD skin gross lesions in the head, dorsal fin, flukes, and caudal peduncle (Figures 2D, E), as well as old injuries likely related to fisheries interactions. The temporal progression and severity of skin lesions in this individual were evident until his death (in 2022) (Table 2 and Figures 2B–D). Histological analyses of biopsied skin revealed granulomatous, nodular, multifocal dermatitis with ulceration and epithelial hyperplasia as well infiltration of neutrophils, macrophages, and giant cells but scarce lymphocytes and plasmatic cells infiltration. The Methenamine silver stain evidenced multiple deep blue round-shaped structures of 6 – 15 μm in diameter interconnected by short tubular structures, which are typical of *L. loboi* (Figure 2F).

Coefficient of association in individuals affected by LLD

Overall, COA values (i.e., among all identified individuals within each study area) were generally low ($\bar{x} = 0.15 \pm 0.14$SD), and in the ALV population (the only with LLD), from the 53 542 possible combinations, only 237 pairs (0.04%) were non-random.

TABLE 1 Surveys and sightings of coastal bottlenose dolphins with and without gross LLD skin lesions at Veracruz, Gulf of Mexico.

Site	Period	Surveys	Number of schools	Dorsal fin photographs	Identified individuals	LLD individuals
Nautla	2002 – 2003	26	25	1 135	160	0
Alvarado	2002 – 2003	26	35	950	65	3
VRS*	2005 – 2007	33	45	3 205	100	0
Alvarado	2006 – 2010	84	260	8 227	210	3
	Total	169	365	13 517	524	3

*Veracruz Reef System.

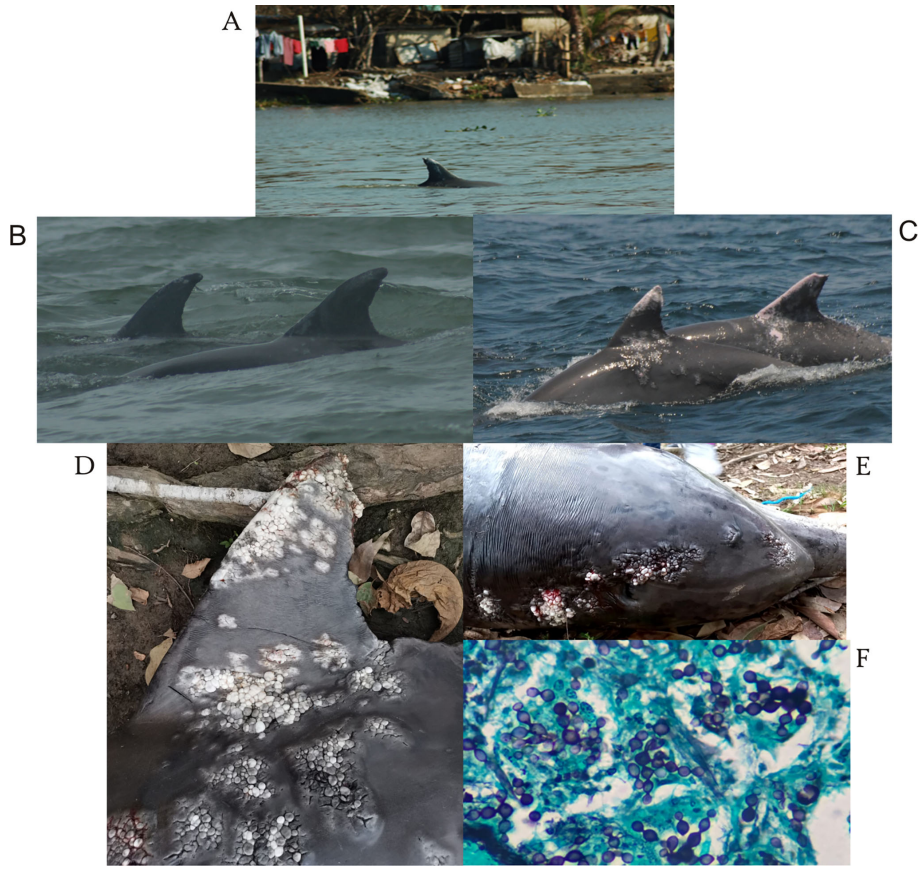

FIGURE 2
Wild-bottlenose dolphins from Alvarado affected by LLD. **(A)** Presence of individual EN05 at Alvarado lagoon close to rural communities exhibiting skin gross lesion possible related to LLD (2006), **(B)** pair of males, EN10 (right) and TN15 (left) with early LLD gross lesions in dorsal fins and dorsum (2006), **(C)** re-sighting of individual EN10 (left) and TN15 (right) with progression of skin lesions in dorsal fins and lateral flanks (2019), **(D)** dorsal fin of individual EN10 stranded at Papaloapan River with severe skin gross lesions, **(E)** multiple nodules ulcerated in head, anterior dorsum near to blowhole and lateral flank (left), **(F)** Microscopic image of dermal tissue of individual EN10. Note presence of rounded deep purple structures connected by a tube-shaped forms, typical of fungus *Lacazia loboi*. Methenamine Silver stain. Magnification, 100x.

From the latter, 11% had low or very low association values (<0.4), 68% were moderate (0.4 – 0.6), and the rest were high (7%, 0.6 – 0.8) or very high (3%, 0.8 – 1.0). Specific COA among males affected by LLD (EN05, EN10, and TN10) yielded high values and thus stable associations ($\bar{x} = 0.64 \pm 0.17$SD), particularly between EN10-TN15 (0.80 from 2003 to 2010) (Figures 2B, C). Individuals EN05 and TN15 were also paired with LLD-negative individuals within the population with higher-than-average association rates (e.g., a possible female named TN10, COA= 0.73 from 2006 to 2010, and other adult individual of undetermined sex named UP22, COA= 0.35, from 2007 to 2010). Therefore, mean COA values for non-null paired combinations of all individuals

TABLE 2 Temporal progression of gross skin lesions (%) linked to LLD in dorsal fins of photo-identified bottlenose dolphins from Alvarado area.

	Right side (%)			Left side (%)		
Individual	**Initial**	**Final**	**Period**	**Initial**	**Final**	**Period (y)**
EN05	2.2	10.5	2003 – 2006	2.6	4.9	2003 – 2006
EN10	10.5	15.3	2003 – 2009	3.8	9.5	2003 – 2010
					66.3	2019
					70.9*	2022
TN15	2.6	22.2	2003 – 2009	9.3	15.9	2006 – 2010

*at time of death.

identified on multiple occasions within the same study area (i.e., ALV) were similar to the overall mean for two of the three individuals (EN10 and TN15) with LLD lesions ($\bar{x}_{EN10}$ = 0.15 ± 0.11SD, $\bar{x}_{TN15}$ = 0.15 ± 0.12SD, $p > 0.05$), but it was very low in individual EN05 ($\bar{x}_{EN05}$ = 0.06 ± 0.12SD).

Discussion

Our study confirmed the presence of LLD in *T. truncatus* at the SWGM, supporting the value of photo-identification surveys as a non-invasive research tool to monitor skin gross lesions progression and histological analyses in stranded individuals as an alternative for epidemiological surveillance studies contributing to health assessment programs in Veracruz, Mexico.

The overall prevalence of LLD in *T. truncatus* from SWGM, which only included individuals from ALV, was within of the ranges observed in other coastal dolphins from the east and west coasts of Florida (<1 to 16.9%) (Wagner et al., 2003; Murdoch et al., 2008), as well *Tursiops* spp. from South America, such as Ecuador (2.33 – 14.3%) (Van Bressem et al., 2015; Félix et al., 2019), but considerably lower than populations from Brazil (9 – 16.7%; Daura-Jorge and Simões-Lopes, 2011; Van Bressem et al., 2015), Costa Rica (13.2 – 16.1%; Bessesen et al., 2014) and cetaceans from Japan (15.8 – 23.1%; Van Bressem et al., 2013), the Indian Ocean (8.4%; Kiszka et al., 2009), and Guerrero, Mexico (9.8%; Ramos et al., 2018). Such geographical and temporal differences are expected for infectious diseases both in human and wild animal populations due to their relation to environmental, ecological, and socio-economic factors (Daszak et al., 2001; Jones et al., 2008), including mycotic diseases like LLD (Seyedmousavi et al., 2015; Gnat et al., 2021).

Studies of LLD in *T. truncatus* are mostly from inshore (coastal and lagoon) populations, and diagnosis is often limited or inconclusive because samples are hard to collect (Van Bressem et al., 2007; Van Bressem et al., 2009b; Kiszka et al., 2009; Ramos et al., 2018). In fact, other photo-ID studies on the species at the north of our study area (i.e., Lobos-Reef System to Nautla, see Figure 1), found no evidence of LLD skin lesions in the 85 photo-identified individuals during 27 surveys in 2016 (Alvízar-Cruz, 2018). However, the presence of LLD in *T. truncatus* at the SWGM during the current research, even at a low prevalence reflects population vulnerability and potentially subjacent health problems (e.g., immunosuppression) in individuals that should be explored.

In cetaceans, LLD susceptibility has been associated to chemical pollution (agricultural, industrial contaminants) that reduces immunological efficiency and facilitates opportunistic infections (Van Bressem et al., 2009b; Reif et al., 2006; Reif et al., 2009; Van Bressem et al., 2009a; Bossart et al., 2019). However, it is not clear whether other chemical and physical characteristics of the habitat (e.g., lower salinity and warmer marine conditions) may favor susceptibility among small cetaceans populations (Reif et al., 2006; Bessesen et al., 2014; Van Bressem et al., 2015; Ramos et al., 2018). All our study areas are characterized by freshwater intrusion derived from the influence of rivers that flow near the coastal habitat of *T. truncatus* at SWGM, particularly, the ALV region, having the second largest hydrographic basin (354 km) in Mexico (Morteo et al., 2019). Toxicological studies have identified organochlorine pesticides, aromatic hydrocarbons, and heavy metals (Pb, Hg) from petrogenic origin (Vázquez-Botello et al., 2018) in both fish and hair from local human communities, especially at the margins of the Papaloapan River in ALV (Guentzel et al., 2007). The ALV *T. truncatus* population is mostly composed of small and single-sex groups, with very fluid and open affiliations and generally low levels of association (Morteo et al., 2014; García-Vital et al., 2015), with a core community of resident individuals compared to Nautla and the VRS (Bolaños-Jiménez et al., 2021; Bolaños-Jiménez et al., 2022). However, coefficients of association among infected *T. truncatus* from ALV were high in contrast to non-infected individuals. It is possible that temporal and spatial exposure to chemical pollutants enhanced by population residency and social structure, may favor LLD, similar to what was suggested in resident *T. truncatus* from Mexico and Belize (Ramos et al., 2018). However, in both cases, ecotoxicological studies are necessary to clarify the relation between habitat pollution and disease epidemiology.

In small cetaceans, chronic stress increases vulnerability to LLD and other skin diseases (Reif et al., 2009; Van Bressem et al., 2013). Different immune response suggests sex-biased susceptibility (Van Bressem et al., 2018), as LLD severity and the highest prevalence has been reported in males of the Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) (Reif et al., 2009; Van Bressem et al., 2013), and *T. truncatus* from Mexico (Tenorio-Osorio, 2015) and Ecuador (Félix et al., 2019). In these last cases, 100% and 86% of individuals with LLD were adult males, closely associated in small groups (pairs) with higher association coefficients. These findings may imply social vulnerability related to sex (males) and age class structure (adults) in *T. truncatus* at the SWGM.

The close associations among LLD positive *T. truncatus* at ALV suggest horizontal transmission during their physical interactions, given the typical social affiliations of the community (Tenorio-Osorio, 2015; Morteo et al., 2017), and the likelihood to present superficial dermal wounds over their development (Paniz-Mondolfi et al., 2007; Murdoch et al., 2008; Kiszka et al., 2009; Murdoch et al., 2010; Daura-Jorge and Simões-Lopes, 2011). Furthermore, LLD in our study suggests a geographical pattern (Daura-Jorge and Simões-Lopes, 2011; Félix et al., 2019); for instance, Ruiz-Hernández (2014) and Morteo et al. (2019) already showed limited individual exchange (<1 individual y^{-1}) among the SWGM. Therefore, behavioral

patterns (e.g., social and feeding) and individual residency, combined with local habitat characteristics (e.g., pollution) could be limiting contact among infected individuals and preventing LLD transmission to *T. truncatus* from Nautla and VRS. However, extended epidemiological efforts in communities (dead and alive) are warranted to explore this hypothesis, as well as the potential differences in age classes and sex.

LLD skin gross lesions described in *T. truncatus* from ALV are similar to macroscopic findings reported in small cetaceans worldwide (Moreno et al., 2008; Murdoch et al., 2008; Daura-Jorge and Simões-Lopes, 2011; Van Bressem et al., 2013; Van Bressem et al., 2015). Also, microscopic characteristics of these lesions in the stranded dolphin at Papaloapan River matched the histological description in the skin of small cetaceans affected by LLD (Migaki et al., 1971; Haubold et al., 2000; Moreno et al., 2008; Rotstein et al., 2009; Ueda et al., 2013). Thus, according to macroscopic and microscopic findings in skin of individual EN10 and due to the lack of molecular evidence, it is plausible that the other two males in ALV were affected by *L. loboi*. Admittedly, other nodular and granulomatous progressive skin diseases reported in small cetaceans, including *Tursiops* sp., that are associated to fungus such as *Fusarium* spp., *Paracoccidioides brasiliensis* and *Trichophyton* spp., and bacteria like *Streptococcus iniae* may be causing these lesions (Van Bressem et al., 2008; Van Bressem et al., 2013; Vilela et al., 2016).

Progressive development of LLD skin gross lesions progression in cetaceans is variable within similar and different species, ranging from 5 to 15 y (Murdoch et al., 2008; Ramos et al., 2018). This reflects the chronicity of long-lived species exposed to LLD, linked to immune system activation and the inability to eliminate prolonged inflammation that could favor the persistency and progression of the disease (Bossart et al., 2019). This seems to be the case for the photographed dead male (EN10) found at the Papaloapan River over the course of 20 y. This reflects the chronicity of LLD in small cetaceans and an increase of skin gross lesion progression during aging, possibly linked to immune senescence, implying a limited disease control and vulnerability to opportunistic disease infections (Venn-Watson et al., 2011; Venn-Watson et al., 2020) like LLD. In some cases, this may be aggravated by subjacent conditions, such as immunosuppression by chronic exposure to heavy metals and hydrocarbons (De Guise et al., 2002; Desforges et al., 2016) present in ALV (Guentzel et al., 2007; Vázquez-Botello et al., 2018) and could influence LLD skin gross lesion progression in individuals.

Increasing LLD prevalence in dolphin populations could represent a potential threat for long-term survival (Van Bressem et al., 2015); however, this does not seem to be the case within the SWGM, since within the timeframe of the photo-ID surveys (i.e., 2002 – 2003, 2005 – 2007, and 2006 – 2010) there were no new infected individuals. Thus, social structure, behavior, and residency seem to conform to a positive mechanism for disease control through potential social isolation and the natural death of affected *T. truncatus*. It is also possible that LLD negative (not gross skin lesions) individuals such as UP22 have higher contagion risk because of their regular and long-lasting associations with infected individuals. In fact, individual EN10 was spotted in subsequent surveys (2019) with a new and unidentified individual with LLD skin lesions. Therefore, intrusion and contact with new individuals could represent another source of LLD exposure.

Furthermore, attention must be drawn to the fact that marine pollution remains a health risk for *T. truncatus* in the Gulf of Mexico, due to the elevated level of oil-related activities (Vázquez-Botello et al., 2004, Vázquez-Botello et al., 2018) and the explosion of the Deepwater Horizon oil spill in 2010 with short- and long-term consequences in the health and survival of *T. truncatus* in the northern Gulf of Mexico (Schwacke et al., 2014; De Guise et al., 2017). These and other human activities (e.g., interaction with fisheries) have resulted in injuries in up to 11.5% of the individuals at SWGM (Morteo et al., 2017) and may play an important role in the onset of skin diseases (Rowe et al., 2010) like LLD, which is a typical cutaneous disease from rural, humid and tropical environments with abundant vegetation and soil; these last two factors are considered potential sources for human infection in localities near to rivers and creeks, including local marine wildlife such as *T. truncatus*. The latter suggests *L. loboi* as a hydrophilic fungus (Lupi et al., 2005; Queiroz-Telles et al., 2011) that could be already present within the tropical and humid environment of the ALV lagoon (García, 1973). Potential LLD cases reported in *T. truncatus* from ALV add to the reports of LLD diagnosed in humans from the Gulf of Mexico (Pech-Ortiz et al., 2020). Therefore, *T. truncatus* could be considered as local sentinel of LLD disease risk for the rural ALV community, reinforcing the vulnerability and interconnectivity between humans, wildlife, and environmental health in low-income countries such as Mexico, where 40% of infectious diseases (i.e., zoonoses) emerged from animals (Grace et al., 2012). Since exploitation of coastal marine resources is crucial for human livelihood in Veracruz (Sánchez-Gil et al., 2004), and both species have overlapping diets (Chávez-Martínez et al., 2022), LLD presence in the ALV marine ecosystem is a potential risk to public health.

Additionally, long-term multidisciplinary studies in *T. truncatus* at the SWGM are necessary to define the interindividual and environmental characteristics involved in LLD susceptibility. Molecular diagnosis of the agent linked to LLD and disease surveillance related to climate change and other skin problems (e.g., trauma) at ALV are warranted, while studying the epidemiology and diagnosis of LLD in rural communities from the ALV lagoon.

Finally, we highlight the importance of a regional marine mammal stranding database that helps identifying health and survival threats, and their relation to environmental hazards at the SWGM (Chan et al., 2017) using both traditional veterinary

protocols (e.g., necropsy) and near future modern technology (e.g., virtopsy) to improve individual diagnosis (Tsui et al., 2020) and hence to implement appropriate conservation strategies based in health programs for *T. truncatus* at Veracruz.

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

CG - Conceptualization, Methodology, Manuscript Writing and reviewing. MT-O - Data collection, Sample processing, Data analysis, Manuscript Writing. IH-C - Conceptualization, Methodology, Data collection, Data analysis, Manuscript reviewing. CD-A - Methodology, Data collection, Data analysis, Manuscript reviewing. EM - Conceptualization, Funds acquisition, Project manager, Methodology, Data collection, Manuscript writing and reviewing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Microplastics in gastric samples from common bottlenose dolphins (*Tursiops truncatus*) residing in Sarasota Bay FL (USA)

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The oceans contain trillions of plastic particles, mostly microplastics (i.e., particles < 5 mm diameter; 92.4% of plastic particles), which have been detected in organisms at all levels of the marine food web. The ubiquity of marine plastic debris has created a monumental environmental pollution problem with extensive public health consequences, as more than 40% of the world's population lives near the coast and shares coastal resources. For decades, common bottlenose dolphins (*Tursiops truncatus*) have been used as sentinels of marine pollution risks for coastal communities that rely on seafood. Recently, prevalent phthalate exposure was documented in bottlenose dolphins residing in Sarasota Bay, FL, at concentrations exceeding those of human reference populations. While the source of their exposure is uncertain, the types of compounds detected suggest a plastic origin. The objective of this study was to screen for plastic ingestion among free-ranging dolphins in Sarasota Bay using gastric samples collected during catch-and-release health assessments. Gastric samples were collected from seven live bottlenose dolphins in 2019, and suspected microplastic particles were detected in all samples. The number of particles per sample ranged from <10 to >100, and the most common types were transparent films and white foams. Similar to other marine mammal studies, fibers were also present. Given that dolphins are likely exposed to microplastics *via* contaminated prey, findings from this and additional studies will help to evaluate the potential of contaminated seafood as an additional source of microplastic exposure for humans, as well as help to inform intervention and risk communication needs regarding seafood safety.

KEYWORDS

plastic, contaminant, pollution, cetacean, marine mammal

Introduction

Plastics are of increasing concern to environmental health because of their ubiquitous presence in the global oceans (Eriksen et al., 2014), combined with the fact that plastic waste is slow to degrade and therefore persists in the environment (Andrady, 2011). Geyer et al. (2017) estimated a global plastic production of 380 million tons annually, of which approximately 60% ends up in landfills or the natural environment. Marine plastic debris is now recognized as a pollutant of international concern due to impacts on wildlife and seafood safety, stimulating the initiation of a formal plan at the 2015 G7 Summit targeting improvements in the management and reduction of marine litter G-7 Leaders' Declaration (2015).

Factors impacting the degree of marine microplastic pollution include land use (i.e., commercial, industrial, transport), proximity to urban centers, current and water flow, polymer composition, particle characteristics (e.g., density, size; Su et al., 2020), and wastewater treatment practices (Freeman et al., 2020). Coastal microplastic 'hotspots' are suspected to occur in urbanized areas near industrial and commercial activities, especially those located downstream of large rivers and waterways (Su et al., 2020). Marine plastic debris is categorized by size (Andrady, 2011). Macro- and mesoplastics (≥ 5mm diameter) often enter the marine environment directly as waste, while marine microplastics (<5mm diameter) may come from wastewater treatment facilities or fragmentation of larger plastic items (Cole et al., 2011; Geyer et al., 2017). Eriksen et al. (2014) estimated that the oceans contain more than 5.25 trillion plastic particles, of which 92.4% are microplastics.

Previous studies have demonstrated microplastic ingestion by filter-feeding and lower-trophic level organisms (Autian, 1973; Farrell and Nelson, 2013; Fossi et al., 2014) as well as translocation of microspheres (1 to 5 μm) into muscle tissue (Zeytin et al., 2020), suggesting the potential for trophic transfer to apex predators. In fact, microplastics have been detected in the stomachs, intestines, and feces of many marine mammal species (Zantis et al., 2021; Table 1), but the health consequences of microplastic exposure are still uncertain. *In vitro* and *in vivo* laboratory studies have linked microplastic exposure with gastrointestinal inflammation, altered gut microbiota, metabolic changes, antibiotic resistance, and oxidative stress, among other impacts from particles ranging from nano to 250 μm (Lu et al., 2018; Jin et al., 2019; González-Acedo et al., 2021).

TABLE 1 Recent studies documenting microplastic ingestion in cetaceans.

Species	Disposition	Sample(s)	Microplastic Types	Studies
Humpback Whale *(Megaptera novaeangliae)*	stranded	stomach, intestine	sheets, fibers, fragments	Besseling et al., 2015
Killer Whale *(Orcinus orca)*	stranded & bycatch	digestive tract	no specific details provided	Lusher et al., 2018
Cuvier's Beaked Whale *(Ziphius cavirostris)*	stranded & bycatch	digestive tract	no specific details provided	Lusher et al., 2018
True's Beaked Whale *(Mesoplodon mirus)*	stranded & bycatch	digestive tract	fibers, fragments, films	Lusher et al., 2015; Lusher et al., 2018
Common Dolphin *(Delphinus delphis)*	stranded & bycatch	stomach, full digestive tract	fibers, fragments, beads	Hernandez-Gonzalez et al., 2018; Lusher et al., 2018; Nelms et al., 2019
Harbor Porpoise *(Phocoena phocoena)*	stranded & bycatch	full digestive tract, intestine	fibers, fragments	Lusher et al., 2018; Van Franeker et al., 2018; Nelms et al., 2019; Philipp et al., 2021
Risso's Dolphin *(Grampus griseus)*	stranded	full digestive tract	fibers, fragments	Nelms et al., 2019
Pygmy Sperm Whale *(Kogia breviceps)*	stranded	full digestive tract	fibers, fragments	Nelms et al., 2019
White-Beaked Dolphin *(Lagenorhynchus albirostris)*	stranded	full digestive tract	fibers, fragments	Nelms et al., 2019
Atlantic White-Sided Dolphin *(Lagenorhynchus acutus)*	stranded	full digestive tract	fibers, fragments	Nelms et al., 2019
Striped Dolphin *(Stenella coeruleoalba)*	stranded	full digestive tract	fibers, fragments	Lusher et al., 2018; Nelms et al., 2019
Bottlenose Dolphin *(Tursiops truncatus)*	stranded & bycatch	full digestive tract, stomach and intestinal subsections	fibers, fragments, films, foams	Lusher et al., 2018; Nelms et al., 2019; Battaglia et al., 2020
Beluga Whale *(Delphinapterus leucas)*	subsistence harvest	stomach, intestine, feces	fibers, fragments	Moore et al., 2020
East Asian Finless Porpoise *(Neophocaena asiaeorientalis sunameri)*	bycatch	intestine	fibers, sheets, fragments, foam	Xiong et al., 2018
Indo-Pacific Humpbacked Dolphin *(Sousa chinensis)*	stranded	intestinal subsections	fibers, fragments, flakes	Zhu et al., 2019

Recent evidence from Leslie et al. (2022) suggest that many organ systems can be exposed as plastic polymers can enter and be transported by the bloodstream. The toxic threat of microplastics may be two-fold due to both the plastic particle itself and endocrine disrupting chemical additives (e.g., phthalate acid esters, "phthalates"; Rochman et al., 2019). In fact, plasticizers compose up to 70% of some plastics, and it is anticipated that the demand and market for these additives will continue to grow (Benjamin et al., 2017).

Sarasota Bay, located on the central west coast of Florida, is a semi-closed estuarine system with minimal tidal exchange (SBEP, 2014). As an urban watershed, this region houses multiple residential, industrial, and commercial centers (USF, 2020). Bottlenose dolphins in this area have been the focus of numerous studies since 1970, including recent investigations of phthalate exposure (Hart et al., 2018; Hart et al., 2020; Dziobak et al., 2021). These studies found prevalent exposure among live dolphins sampled during 2010-2019 (75%; n=51; Hart et al., 2020; Dziobak et al., 2021) and heightened exposure to a metabolite that suggests a plastic origin (mono-(2-ethylhexyl) phthalate, MEHP; Hart et al., 2020). We suspect incidental ingestion of microplastics could be a source of phthalate exposure for bottlenose dolphins, evidenced by studies of phthalate exposure in laboratory mice that were fed plastic particles laced with chemical additives (Deng et al., 2020). Additionally, previous studies have demonstrated that plastic particles in general are highly resistant to digestive juices (Stock et al., 2020), suggesting the potential for chronic exposure.

Prior studies investigating microplastic exposure and ingestion among cetaceans (i.e., dolphins, porpoises, whales) have relied primarily upon investigations of gastrointestinal tracts from dead stranded, or bycaught animals (Lusher et al., 2015; Lusher et al., 2018; Nelms et al., 2019; Zantis et al., 2021; Table 1), as well as computer simulation models that predicted geospatial overlap of microplastic contaminated waters and large-whale foraging areas (Fossi et al., 2017). Additionally, most of the studies on marine mammal exposure to microplastics have focused on pinniped species and regions in Europe (Hudak and Sette, 2019; Zantis et al., 2021). To our knowledge, this the first study to screen for ingested microplastics in free-ranging bottlenose dolphins, and one of the few studies characterizing marine mammal microplastic exposure in the southeastern United States. Using gastric samples collected from long-term resident bottlenose dolphins inhabiting an urban estuary (i.e., Sarasota Bay, Florida), the objective of this descriptive study was to screen for evidence of microplastic ingestion and evaluate the characteristics of ingested microplastics (e.g., shape, color, quantity, size). Findings from this study will provide a foundation for a future systematic assessment of ingested microplastics in Sarasota Bay dolphins and contribute to a larger effort to understand potential sources of phthalate exposure among Sarasota Bay dolphins.

Methods

Study population and sample collection

Bottlenose dolphins have been the focus of extensive study in Sarasota Bay, FL since 1970 to understand the behavior, movement patterns, and health of the approximately 170 individuals that inhabit the region year-round, over decades and across generations. Using well-established catch-and-release techniques developed and refined over more than 50 years, dolphins were encircled by a net and temporarily restrained to collect biological samples indicative of an individual's health (Wells et al., 2004). Gastric samples were collected by passing a small tube (9-15mm diameter) through the esophagus into the stomach (Twiner et al., 2011), and gastric fluid was stored in amber glass jars and frozen at -20°C until analysis. Approximately 20mL of gastric fluid was collected from each dolphin. Environmental blanks were collected by passing deionized water through the stomach tubes prior to the collection of dolphin samples. One environmental blank was collected per sampled dolphin to control for potential field contamination. All samples for this study came from bottlenose dolphin health assessments that were conducted during June 3 -June 14, 2019. Samples for this study were collected under Scientific Research Permit No. 20455 from the National Oceanic and Atmospheric Administration's (NOAA) National Marine Fisheries Service (NMFS). All catch-and-release and sampling methodologies for the health assessments were reviewed and approved by Mote Marine Laboratory's Institutional Animal Care and Use Committee (IACUC).

Sample analysis

For the processing of microplastics, bottlenose dolphin gastric samples were emptied into a 250 mL glass beaker. Organic (non-plastic) material in the samples was digested by adding 150 mL of KOH (10% solution) and incubated at 60°C for 24 hours (Valton et al., 2014). Following digestion, samples were vacuum filtered onto GF/A 1.6 µm glass fiber filters in a fume hood. Samples were left to dry in covered glass petri dishes. Particles of at least 35 µm in size were characterized visually using a dissection microscope (Leica EZ4, magnification 8-35x) according to physical attributes including shape (e.g., fiber, film, fragment, foam), surface texture, and color (e.g., transparent, blue, black; Shim et al., 2017). Various parameters were examined to indicate potential plastic material. Suspected plastic fibers were indicated by a smooth, uniform surface with a length that exceeded the width (Lusher et al., 2020). Suspected plastic fragments were indicated by smooth or angular edges that appeared to be broken from a larger piece of debris (Lusher et al., 2020). Fragments were further categorized as films if they were flexible and able to be folded, or foams if they were distorted

when physically handled, but eventually returned to their original shape (Lusher et al., 2020). Particles (at least 100 µm in size) were tested with a hot needle (250°C) and suspected to be of plastic origin if the needle melted or left a mark on the particle surface (De Witte et al., 2014; Devriese et al., 2015). A subsample of suspected plastic particles ranging between 500 µm and 5mm in diameter was further examined by Fourier Transform Infrared (FTIR) spectroscopy to determine composition. Particles were analyzed using a Nicolet iS20 FTIR (Thermo Scientific) with diamond attenuated total reflectance (ATR). Recorded spectra were processed and matched to a reference spectral library using Open Specy Software (Cowger et al., 2021). Polymers were noted if the spectral match was at least 50%.

Quality assurance/quality control

Rigorous precautions were taken while handling and processing samples. A 100% cotton lab coat and nitrile gloves were worn during laboratory analyses. All tools and glassware were carefully rinsed with distilled water that was filtered through 90 mm GF/A 1.6 µm glass fiber filters. For QA/QC purposes, one lab/procedural blank extraction without tissue was performed simultaneously with each set of sample digestions to correct for potential procedural contamination, and three positive controls with commercially purchased polyethylene, polystyrene, and polyester microplastic particles were used to determine recovery efficiency. Mean recovery percentages were 90% for film, 87% for foam, and 90% for fibers. During the dolphin health assessments, environmental blanks were collected concurrently with gastric fluid, using the same sampling methods and equipment. Also, the sample collection and processing teams wore green cotton shirts, and the caps for the glass sampling tubes were only removed once the gastric sample was collected from the dolphin.

Data analysis

Methods to characterize microplastic exposure in bottlenose dolphins followed the methods of Nelms et al. (2019) and best practice recommendations by Zantis et al. (2021). Briefly, we estimated the number of suspected microplastic particles per gastric sample, calculated the proportion of samples containing at least one suspected plastic particle, and identified key physical characteristics (e.g., shape, color). On a random subset of suspected plastic particle types (e.g., films, foams, fibers), we assessed polymer composition *via* FTIR and measured particle dimensions. As this study was exploratory and sampling volumes were not standardized, systematic exposure assessments were not performed for each individual. Further, given the small sample size, statistical comparisons between age groups or sexes were not performed.

Results

Gastric samples were collected from seven free-ranging, long-term resident bottlenose dolphins during health assessments in June 2019. Four of the dolphins were females, and ages ranged from 4 to 44 years (Table 2). Dolphins were sampled from four locations throughout Sarasota Bay: 1) one dolphin in Palma Sola Bay; 2) one dolphin near Tidy Island; 3) three dolphins near the southern end of Longboat Key; 4) two dolphins near the northern end of Siesta Key (Figure 1; Table 2).

Of the total 92 particle types remaining in samples after digestion and filtration, 62% (n=57) were of suspected plastic origin, based on the hot needle test. All suspected plastic particles examined were less than 5mm in diameter, suggesting microplastic ingestion in bottlenose dolphins. At least one suspected microplastic particle was observed in gastric samples of every dolphin (Table 3). Suspected microplastic quantity was variable across dolphin samples (less than 10 to greater than 100), and particle types included several colors of films, fibers, and foams (Figures 2–4; Table 3). Transparent films and white foams were the most commonly observed particle types in bottlenose dolphin gastric samples (Table 3). FTIR was not conducted on fibers due to their small widths, and many of the white foams were too small to yield conclusive results. However, polymers (e.g., PVC zinc, polyethylene, polyamide) were detected in transparent films, providing evidence of microplastic ingestion in five of the seven dolphins (Table 4).

TABLE 2 Characteristics of bottlenose dolphins sampled in Sarasota Bay, Florida during health assessments (2019).

Dolphin ID	Sample Collection Date	Sex	Age	Sampling Location
F128	6/10/2019	M	27	Siesta Key
F221	6/10/2019	F	14	Siesta Key
F261	6/4/2019	F	44	Tidy Island
F264	6/6/2019	M	11	Longboat Key
F266	6/6/2019	M	12	Longboat Key
F283	6/5/2019	F	4	Palma Sola Bay
F285	6/6/2019	F	11	Longboat Key

M, male; F, female.

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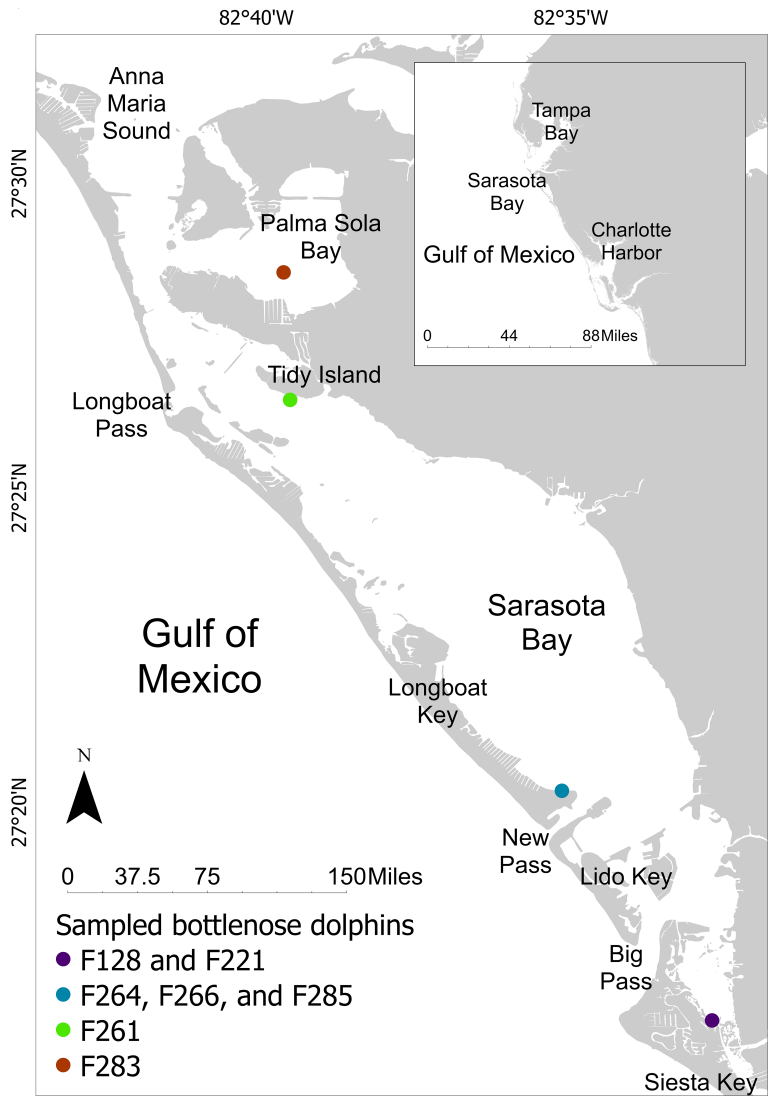

FIGURE 1
Locations of sampling sites for Bottlenose Dolphins Sampled during 2019 Catch-and-Release Health Assessments in Sarasota Bay, Florida.

Suspected plastic particles were also present in all environmental and laboratory/procedural blanks, ranging from one to 12 particles per blank (Tables 3, 4). The majority of suspected plastic particles in the environmental and laboratory blanks were fibers; transparent films and foams were not observed in any of the blanks (Tables 3, 4).

Discussion

Overall, our findings are consistent with previous studies. In a review of 30 publications reporting results of microplastics screening of gastrointestinal tracts (primarily from dead stranded or bycaught cetaceans) and scat (primarily from live pinnipeds), microplastics were highly prevalent (Zantis et al., 2021). In our study, all dolphin samples contained at least one suspected plastic particle, and most contained more than 50 particles. Particle types observed in Sarasota Bay dolphin samples were similar to those reported in previous studies, including fibers, foams, and films. However, previous studies reported a predominance of fibers (Zantis et al., 2021; Table 1), while foams and films dominated the particle types ingested by Sarasota Bay dolphins (Table 3). Our findings agree with previous cetacean studies in which blue and black fibers were commonly observed (Zantis et al., 2021), but the most abundant particle colors in Sarasota Bay dolphin samples were transparent and white.

Given widespread reports of microplastic ingestion among many stranded cetaceans of a variety of species (Table 1), it is not

TABLE 3 Microplastic characteristics in environmental/laboratory blanks and gastric samples collected during catch and release bottlenose dolphin health assessments conducted in Sarasota Bay, FL, USA (June 2019).

Dolphin ID	Sample Type	Estimated Count(all particles)	Clear	White	Blue	Green	Black	Red	Yellow	Purple	Pink	Orange
F128	Gastric	< 20	***Fl***	Fo	F							
	Blank	missing										
F221	Gastric	>100	***Fl***		Fl, F		F					
	Blank	8			F		F		F			
F261	Gastric	50-100		***Fo***	F	F	F					
	Blank	14	F		F		F	F	F	F		
F264	Gastric	>100	Fl	***Fo***	F		F				F	
	Blank	4		F				F				
F266	Gastric	10-50	F		F	F	F					
	Blank	6			F							
F283	Gastric	50-100	***Fl***, F	Fo	F		F	F	F			
	Blank	5			Fl, F		F	F				
F285	Gastric	>100	***Fl***, F		F			F	F			
	Blank	7			F		F					
Lab	Blank 1	7			F		F			F		F
	Blank 2	1			F							
	Blank 3	1						F				

Italic, bold font indicates most common particle type in sample. Fl, film; Fo, foam; F, fiber.
Shaded cells indicate particle type overlap between gastric and blank samples.

entirely surprising to detect evidence of microplastic ingestion in Sarasota Bay dolphins. Although there are no published microplastic surveys for Sarasota Bay, microplastic contamination along the coastal regions of the Gulf of Mexico is among the highest found worldwide, with reported concentrations ranging from 60-1,940 items/kg in sediment, 12-381 particles/L in water, and 1.3-4.7 particles per fish (reviewed by Shruti et al., 2021). Microplastic concentrations in sediments, which serve as a sink for these particles, were strongly influenced by degree of urbanization (Shruti et al., 2021). Further, a study conducted in Tampa Bay, which is directly north of Sarasota Bay, estimated approximately 4 billion particles in the bay (McEachern et al., 2019). As a result, it is reasonable to expect similar levels of contamination in Sarasota Bay.

The differences in particle characteristics observed between Sarasota Bay dolphins and previous cetacean studies could possibly be explained by geography, sample type (gastric fluid vs. intestine), or retention/passage time. For example, Giani et al. (2019) found geographic differences in the composition of microplastic types among fish inhabiting distinct regions of the Mediterranean Sea. Also, given the likelihood for trophic exposure (Nelms et al., 2018), the types of particles observed in Sarasota Bay dolphins could also be attributed to geographic differences in prey distribution. Alternatively, Nelms et al. (2019) note that microplastic distribution is inconsistent across the gastrointestinal tract, resulting in differences that may be dependent on the anatomical location of sampling (e.g., stomach vs. intestine). It also seems plausible that differences in suspected microplastic characteristics observed in this study could be attributed to differences in retention time for different types of microplastics. For example, in a study comparing ingested microplastic characteristics between grey seals (*Halichoerus grypus*) and their prey fish, Nelms et al. (2018) found that microplastic fragments were most common in seal scat samples, while fish gastrointestinal tracts were dominated by fibers. Perhaps foams and fragments are egested quickly, whereas fibers may accumulate in the intestinal tract.

Considerations

One of the limitations in microplastic research is the recurring issue of sample contamination by ambient microplastics during field collection or sample processing (Foekema et al., 2013). Despite our QA/QC efforts, suspected plastic particles (primarily fibers) were observed in nearly all laboratory and environmental blanks, suggesting the possibility of dolphin sample contamination (Tables 3, 4). To help control for contamination levels in the field, environmental blanks were collected and processed in a manner identical to that of the gastric samples. Furthermore, while we were not able to control for synthetic clothing during field collection, the individuals involved in the sample collection and sample processing wore bright green shirts, and green fibers were not prevalent in gastric samples or associated environmental blanks. While overlap in

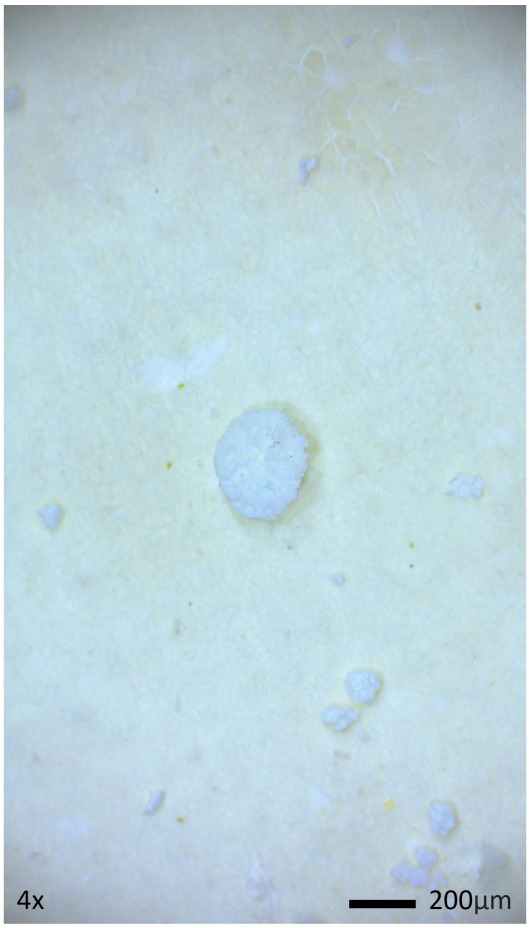

FIGURE 2
Example of White Foam from a Gastric Sample of a Bottlenose Dolphin Sampled in Sarasota Bay, Florida (2019).

fiber color between the gastric samples and both sets of blanks could provide evidence of sample contamination, the rate of contamination in these samples was less than 10% of the total number of suspected microplastics in the gastric samples (Table 3). Additionally, clear films and white foams were not observed in any blank, suggesting true ingestion of these particles by Sarasota bottlenose dolphins.

Although suspected microplastics were observed in every sample collected from Sarasota Bay dolphins, plastic ingestion could only be confirmed in five dolphins due to size limitations of FTIR. Additionally, given that blue and black fibers were observed in gastric and blank samples, laboratory or environmental contamination cannot be ruled out, particularly for dolphin "F266" (Table 3). It is difficult to contextualize microplastic exposure in Sarasota Bay bottlenose dolphins due to differences in sample type and collection methods across research studies, as well as limitations within our own study. For example, considering that our analyses focused only on particles contained in gastric fluid, results presented herein are an underestimate of trophic consumption since particles may adhere to, or be trapped in, tissue lining the gastrointestinal tract. Secondly, understanding individual dosage is complicated by the fact that repeated sampling of individuals is not allowable by permit or feasible since gastric samples were taken opportunistically as other health-related samples and measurements were being obtained. As this is the first study to collect gastric samples from live free-ranging bottlenose dolphins for microplastic screening, future efforts will be made to facilitate microplastic exposure assessments. For example, standardized volumes will be collected to calculate and compare microplastic load between individuals and demographic cohorts (Zantis et al., 2021). Additionally, particle sizes will be systematically measured, and suspected plastic particles identified by the hot needle test will be confirmed using FTIR

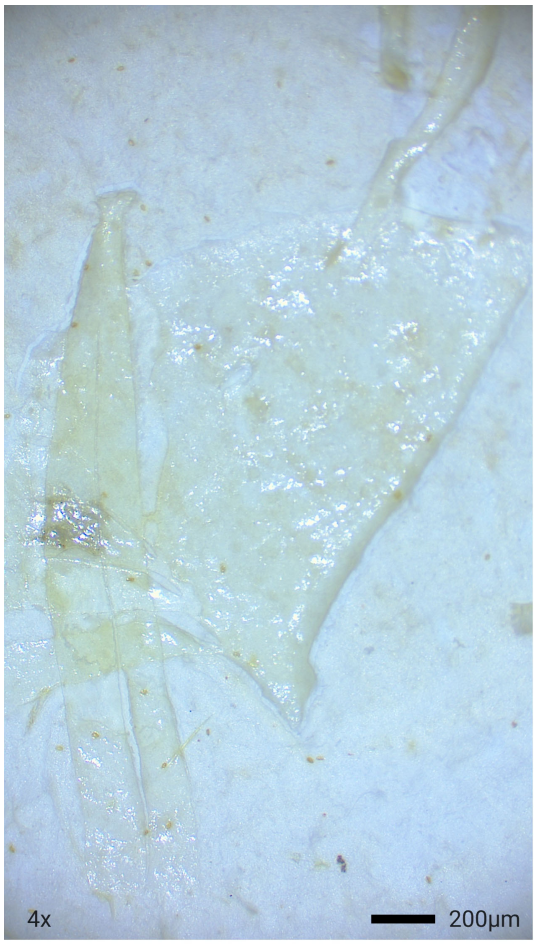

FIGURE 3
Example of Transparent Film from a Gastric Sample of a Bottlenose Dolphin Sampled in Sarasota Bay, Florida (2019).

or Raman spectroscopy, which will enable the determination of polymer composition for suspected particles of a broader size range.

Significance

Our recent studies detected prevalent phthalate exposure among free-ranging bottlenose dolphins in Sarasota Bay, FL (~75%; n=51; 2010-2019; Hart et al., 2020; Dziobak et al., 2021), which is located in the tenth fastest-growing metropolitan region in the United States (U.S. Census Bureau, 2017). The most commonly detected metabolites were monoethyl phthalate (MEP), and mono-(2-ethylhexyl) phthalate (MEHP; Dziobak et al., 2021), which are metabolites of parent compounds commonly added to personal care products and plastic. In fact, Sarasota Bay bottlenose dolphins had significantly higher concentrations of MEHP, the metabolite of di-(2-ethylhexyl) phthalate (DEHP), than human reference populations (Hart et al., 2020). This finding is significant as DEHP is most commonly used in polyvinyl chloride products, food packaging, wire covering, toys, and medical tubing (ATSDR, 2002). While the source of this phthalate exposure is uncertain for bottlenose dolphins, our findings suggest a plastic origin. The link between MEHP detection and DEHP contamination is not clear, but previous studies demonstrated that MEHP concentrations measured in blubber and muscle samples from fin whales and basking sharks were higher in individuals sampled in regions with significantly higher water and plankton microplastic concentrations (Fossi et al., 2012; Fossi et al., 2014).

The ubiquity of plastic has created a monumental environmental pollution problem. The public health consequences of this extensive pollution are significant, as an estimated 41% of the world's population lives within 100 km of the coast (Martínez et al., 2007). As apex predators with a long

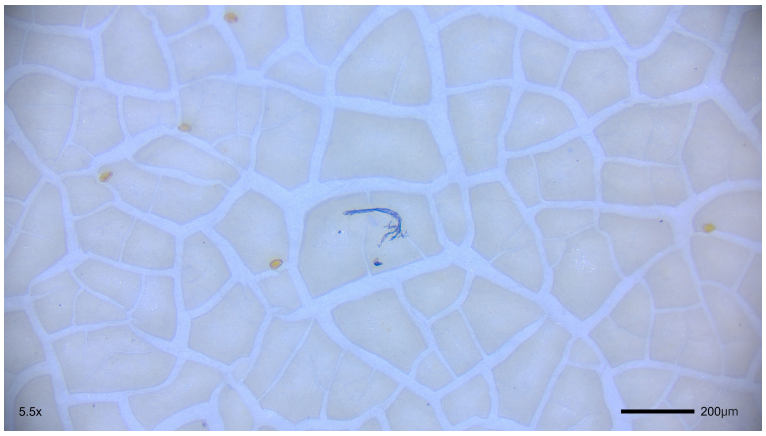

FIGURE 4
Example of Blue Fiber from a Gastric Sample of a Bottlenose Dolphin Sampled in Sarasota Bay, Florida (2019).

TABLE 4 Suspected Plastic Particle Counts, Dimensions, and Composition (determined via FTIR).

Dolphin ID	Fiber (count)	Width Range (µm)	Film (count)	Dimensions (µm)	Polymer	Foam (count)	Dimensions (µm)	Polymer
F128	7	17.61-38.67	13	124.53 x 424.29 829.58 x 309.80	PVC zinc, polyethylene	1	Disintegrated when hot needle applied	N/A
F221	6	15.40-25.62	>100	371.57 x 1.05 mm 816.43 x 1.68 mm 1.05 mm x 2.13 mm	PVC zinc, polyethylene	0	N/A	N/A
F261	7	21.35-26.41	0	N/A	N/A	47	159.73 x 82.311, 541.98 x 268.61	inconclusive
F264	10	21.35-27.01	3	674.35 x 2.90 mm	polyamide	>100	116.22 x 230.63 146.14 x 179.34 432.43 x 404.61	too small
F266	16	17.08-21.35	0	N/A	N/A	0	N/A	N/A
F283	19	17.08-36.74	>50	1.67 mm x 1.20 mm	polyamide	3	209.81 x 264.01	too small
F285	8	24.90	>100	2.34 x 2.53 mm, 459.28 x 1.75 mm	polyethylene	0	N/A	N/A

N/A, not applicable.

lifespan (>60 years; Wells, 2014), year-round resident estuarine bottlenose dolphins can serve as gauges to detect disturbances in their local environment (Wells et al., 2004). This has been demonstrated by epidemiologic studies of exposure to chemical contaminants (e.g., polychlorinated biphenyls, "PCBs"; Wells et al., 2005; Schwacke et al., 2012), toxins produced during harmful algal blooms (Twiner et al., 2011), and oil-associated compounds (Schwacke et al., 2014; Smith et al., 2017), making them excellent sentinels of environmental contaminant exposure for human communities, particularly coastal communities that rely on seafood as a primary source of food or economic export (Backer et al., 2019). In fact, Backer et al. (2019) suggest that because of trophic concurrence, dolphins can warn of local environmental pollution risks for coastal human populations. Findings from this research may assist with efforts to monitor seafood contamination and help to inform intervention and risk communication needs regarding seafood safety.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The datasets generated for this study can be found in the 4TU.Research Data international data repository for science, engineering, and design. https://doi.org/10.4121/19768477.v1.

Ethics statement

The animal study was reviewed and approved by Mote Marine Laboratory's Institutional Animal Care and Use Committee (IACUC). Samples for this study were collected under Scientific Research Permit No. 20455 from the National Oceanic and Atmospheric Administration's (NOAA) National Marine Fisheries Service (NMFS).

Author contributions

Conceptualizaton: LH. Data Curation: MD, BE, RW. Formal Analysis: MD, BE, LH. Funding Acquisition: LH, RW. Investigation: MD, RW, LH. Methodology: MD, BE, LH, JW. Project Administration: LH, RW. Resources: BE, JW, RW. Supervision: JW, BE, RW, LH. Validation: MD, BE. Visualization: MD, LH. Writing Original Draft: LH, MD. Writing Review and Editing: RW, BE, JW. All authors contributed to the article and approved the submitted version.

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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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The endoparasite *Perkinsus olseni* affecting the Mediterranean mussels (*Mytilus galloprovincialis*) in the Italian and Spanish waters: A new possible threat for mussel aquaculture and wild animal population

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Dinoflagellates belonging to the *Perkinsus* genus are OIE (World Organization for animal Health)-listed pathogens extremely virulent for clams and oysters in many marine ecosystems throughout the world. During the monitoring activities of the Mediterranean mussel (*Mytilus galloprovincialis*) in Campania region (Italy), the presence of typical trophozoites of *Perkinsus* sp. was observed in mussels from farms and natural banks. Simultaneously, following mussel mortality in the Spanish waters of Catalonia, histopathological studies revealed the presence of the same parasite. Although perkinsosis is an endemic disease in clams in Italy (with prevalence from 40 to 80%), there are no reports to date of its presence in Mediterranean mussels and of the effect on this species. For this study, histopathology, Ray's Fluid Thioglycollate Medium (RFTM), and molecular diagnostics with conventional Polimerase Chain Reaction (PCR) and qPCR were performed. In samples from Italy, histopathology in the mussel from one farm revealed a prevalence of 26% in February 2019, 40% in February 2020, 16% in November 2020, and 23% in April 2021. In a natural bank, *Perkinsus* was also detected in May 2020 but in lower prevalence. In Spain, in July 2020, the presence of the parasite was 20% in one site and 10% in a second site and related to animal mortality. In both areas, *Perkinsus* sp. elicited multiple inflammatory capsules of different size or infiltrates at the level of the digestive gland and gonad. Molecular diagnostics of the Internal Transcriber Spacer (ITS) region of the rDNA (ITS1, 5.8S, and ITS2) showed a 97% similarity of *P. olseni* from Italy with samples from New Zealand, Australia, and Uruguay and in bivalves such as *Pitar rostrata*, *Astrovenus* sp., and *Haliotis* sp., whereas in Spain the identity was 99% samples from South Korean venerids such as *Anadara granosa*. Phylogenetic analysis group together *P. olseni* from Italian and Spanish mussels but place them distant from other *P. olseni* described in the clams from Europe (Italy, France, and Spain). Direct impact of transboundary animal diseases in aquaculture constitutes a serious consequence for export living animals and their products, as well for international trade. This

compromises food security, also causing a high socioeconomic impact on aquaculture exporting nations. *P. olseni* is a generalist pathogen able to infect different bivalve species, possibly passing from clams to oysters and mussels. Recognized international organizations should take this into account in the view of possible cross-infection. Other studies are needed to define pathogen virulence in this species.

KEYWORDS

Perkinsus, TAADs, mussel, dinoflagellate, emerging pathogen

1 Introduction

In the past years, infectious diseases are emerging significantly in marine and freshwater environments. The elements involved in this emergence are different. Cultivated animals are involved in the global trading, facilitating the introduction of serious infectious diseases, called transboundary diseases (TD). Several transboundary aquatic animal diseases (TAADS) have swept regions over the past 30 years causing massive economic and social losses, responsible for the introduction, establishment, and spread of pathogens into new geographic areas. Nowadays, there are several international codes of practice and guidelines to reduce the risk of introducing pathogens. WOHA (World Organization of Animal Health) has developed recommendations and protocols in the International Aquatic Animal Health Code, which deals with the health surveillance of aquatic animals (OIE Listed Diseases, 2021).

Emerging disease in mussels has been reported repeatedly in the past years, as in other bivalve species. Recently, the potentially zoonotic bacteria *Nocardia crassotreae* were reported in the Mediterranean mussel *M. galloprovincialis* (Carella et al., 2013; De Vico and Carella, 2019), the OIE listed parasite *Marteilia refringens* have been observed in the area (Carella et al., 2010), and many other emerging disease conditions have been also reported in other bivalve species in the same area (Carella et al., 2013; Carella et al., 2018).

Perkinsosis is an important disease that has been reported worldwide in bivalves and gastropods. *Perkinsus* pathogens can infect a wide range of hosts and possibly are responsible for mortality events for their extensive invasive ability and virulence. Nowadays, seven species within the genus *Perkinsus* have been reported including *P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. mediterraneus*, *P. honshuensis*, and *P. beihaiensis* (Ramilo et al., 2015) with only *P. olseni* and *P. marinus* listed notifiable parasites listed by OIE (OIE Listed Diseases, 2021).

Pathogens can display a highly flexible ranges of hosts, called multi-host or generalist pathogens, or can infect only one or a few related species and called specialist pathogens. *P. olseni* has been reported in 30 mollusc species, bivalves, and gastropods over a wide range of geographical locations (Itoïz et al., 2022). It is generally associated with mass mortality of clams such as Manila clams *Ruditapes philippinarum* in Europe, the venerid clam *R. philippinarum*in Asia, the cockle *Austrovenus stutchburyi* in New Zealand (Dungan et al., 2007), and in the abalone *Haliotis* spp in Australia. Recently, reports of perkinsosis in mussels have been increasing; Itoh et al. (2019) reported *P. beihaiensis* infection in the invasive *M. galloprovincialis* in Japan, whereas Vazquez et al. (2022) reported *P. olseni* in *M. chilensis* in Argentina.

In this study, we report, for the first time, the presence of the parasite *Perkinsus* sp. in the Mediterranean mussel *M. galloprovincialis* in Europe. First detection was in mussels from Italy, in Campania region, in mussel farms from 2019 to 2021 and later in natural beds in 2020. During the study, we also observed the presence of *Perkinsus* sp. like cells in mussel samples from Catalonia (Spain) following a mussel episode of mortality. Within the past few years, more data on the genetic variation within some of the *Perkinsus* species have become available, and many ITS (internal transcribed spaces) regions now described have that allowed to assess intraspecific variation and to compare the dissimilarity of sequence with the differences observed among the *Perkinsus* species. During the surveys, we conducted phylogenetic analyses to estimate the relationships with the group of *Perkinsus* spp. in mussels from Italy and Spain and haplotype characterization along with animal histopathology to define host response and possible *Perkinsus* pathogenicity.

2 Materials and methods

2.1 Sampling of Mediterranean mussels in Italy and Spain

During 2018–2021, a field survey targeting infectious agents of the Mediterranean mussel *Mytilus galloprovincialis* was conducted in which mussels were manually collected in one mussel farm and one natural bank on the north coast of the Campania region (Italy).

In Italy, collections were made in a mussel farm in Campania Region (Naples Bay) in February 2019 (N = 20), February 2020 (N = 20), November 2020 (N = 30), and April 2021 (N = 30). A sampling was performed in a close natural bank in May 2020 (N = 30). Alfacs Bay, located in the south of the Ebro delta (Western Mediterranean), is a shellfish growing area where mussels and oysters are grown in ropes hanging from rafts. Sampling in Alfacs Bay was conducted on 21 July 2020, 2 weeks after the beginning of the mortality event on 6 July 2020 as reported by the mussel farmers. Sampling in Alfacs Bay was conducted in two sites, A (40°37'15.28"N; 0°39'14.58"E) and B (40°37'1.98"N; 0°37'51.66"E) (Figure 1). The mussels were transported to the laboratory alive in isothermal boxes. Prior to

FIGURE 1
Locations of sampling areas in Italy and Spain: Bay of Naples and Bay of Alfacs.

processing, the animals were measured for animal shell length, total weight, and meat weight (MW) according to Galtsoff (1964).

2.2 Light microscopy

For animal histopathology, from each animal, a transverse section including digestive tissue, gill, foot, and gonad was obtained and fixed in Davidson's solution for 72–96h. Fixed tissues were embedded in paraffin blocks and sectioned at 3 µm with a rotary microtome (Bioptica, Italy). Tissue sections were deparaffinised, stained with Carazzi haematoxylin and eosin and a special stain such as Mallory's trichrome (Mazzi, 1977). Digital images and measurements were obtained using an integrated Axioscope A1 (Zeiss, Germany) and camera Axiocam 208.

2.3 PCR and qPCR for *Perkinsus* species identification and presence evaluation

From each specimen, 25–30 mg of gonad, digestive tissue and gills, preserved in TE buffer at −20°C, was taken for total genomic DNA extraction using QIAamp DNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions (tissue protocol). The DNA quality and quantity were measured with the use of a Nanodrop spectrophotometer (Thermo Fisher Scientific) and stored at −80°C for long-term preservation. Primers used in the study are listed in Table 1. More in detail, PCR assays using the generic primers PerkITS750/PerkITS85 (Casas et al., 2002) were carried out first to detect *Perkinsus* spp. in all sampled mussels. PCR reactions were carried out in 50 µl of final volume using the Mastermix GoTaq polymerase (Promega) following the instructions of the manufacturer. A positive control provided by IRTA institute constituted by a clam infected *P. olseni* was included in each reaction along with a negative control (master mix with no DNA). Amplification parameters were performed as follows: An initial denaturation of 4 min at 94°C followed by 35 cycle amplifications (1 min at 94°C, 1 min at 53°C, and 3 min at 68°C) and a final extension of 5 min at 68°C. The resulting PCR products were purified and sent to an external sequencing facility (Eurofins Genomics, Germany).

To better define pathogen presence in the Italian samples, a more sensitive procedure of real-time quantitative PCR (qPCR) was also performed using primers Perk-ITS-qF1/Perk-ITS-qR2 (Ríos et al., 2020) that amplifies the internal transcribed spacer region (ITS-1 and ITS-2) of the gene complex that codes for ribosomal RNAs in *P. olseni*. Wells were filled to a final volume of 10 µl, using 1 µl of DNA, 5 µl of Taq Universal SYBR green mix (Applied Biosystem), 0.5 µl of each primer (10 µM), and 3 µl of distilled water. Amplification was performed under the following conditions: denaturation for 10 min at 95°C, amplification by 40 cycles of 15 s at 95°C and 60 s at 60°C, melting curve evaluation 1 min at 95°C, and increase of 0.5°C each 30 s starting in 60°C, end at 95°C for 15 s. All reactions were performed using two technical replicates.

2.4 Phylogeny and haplotype analysis *of Perkinsus* based on the ribosomal *ITS* region

The resulting ITS chromatograms (648 bp) were analyzed using BioEdit software (v. 7.2). All generated sequences were searched for identity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences were also aligned with

the available sequences for *Perkinsus* spp. found in the GenBank database using the MUSCLE algorithm. Maximum likelihood (ML) analysis was conducted using MEGA version X software (Kumar et al., 2004) with 1,000 replicates for calculating bootstrap values.

For the haplotype network analysis, ITS1 sequences of *Perkinsus* obtained from Campania samples (n = 2) and Catalonia (n = 2) and 163 ITS1 sequences of *P. olseni* with geographic information deposited in GenBank were used. Using MEGA X software, sequence data were aligned by CLUSTAL W (Thompson et al., 1994) at default settings. The haplotype network among 163 ITS1 sequences of *P. olseni* was constructed with the TCS network method (Clement et al., 2000) using PopART (Leigh and Bryant, 2015).

2.5 Ray's fluid thioglycollate medium *RFTM assay*

RFTM has been considered the best assay for *Perkinsus* diagnosis (Ray, 1963). In the mussel farm from Italy, starting from the samples of 2020, aseptically excised small pieces (3–5 mm) of digestive gland, gill, mantle, and muscle were in RFTM supplemented with Chloramphenicol 2,5% w/v and Nystatin (4000 U ml^{-1}) in the dark, at for 6 days at 26°C. After incubations, the tissues were placed on a glass slide, covered by a drop of Lugol's iodine solution, cover-slipped and examined under a light microscope (Zeiss Axioscope 5) at different magnification (4×, 10×, and 20× objectives). The sample resulted positive when blue–black hypnospores were observed.

3 Results

3.1 Animal histopathology

Perkinsosis prevalence in mussels from Italy was variable over the years and seasons by light microscopy (Table 2). The highest prevalence was detected in April 2021 with the 23% of the affected individuals.

In Italy, typical features of *Perkinsus*-like cells with a trophozoite characterized by a large vacuole and eccentric nucleus were generally visible, accompanied by the typical production of many inflammatory capsules of different dimensions and with an infection intensity from moderate to high (Figures 2A–D). Chronic lesions, underlined by the involvement of fibroblast, were observed in samples from April 2021. The parasite developed in the connective tissue in the digestive gland, between the tubules, in the connective stroma in the gonad, and in few cases in the muscle of the foot. Within the capsule were visible haemocytes phagocytising at different phases of development were visible, from multinucleate schizonts from four to eight cells. Apoptotic haemocytes were also visible along with the production of yellowish granules (Figures 2A, C, E). In Spain, sites A and B showed animal mortality, 69% in site A and 17% in the site B. The infection was visible through histopathology in two individuals (2/10) in the site A and in one individual in site B. The inflammatory lesion was intense and infiltrative, spreading in all the digestive tissue, with total disappearance of tissue architecture. Moreover, differently from animals in Italy, trophozoites of *P. olseni* were also observed in the gill, a typical *Perkinsus* spp. tropism, and in the haemocyte vessels (Figure 2F). Clusters of trophozoites were encapsulated in well-circumscribed walls forming a cyst-like structure.

3.2 Disease diagnosis with RFTM, PCR, and qPCR

After 6 days, RFTM-cultivated infected mussel stained with Lugol's iodine exhibited dark blue/black hypnospores in the digestive gland and mantle tissue and rarely in the gill (Figures 3A, B). In mantle, muscle, and digestive gland, hypnospores were observed as clusters when the intensity was low or completely dispersed. Comparison with PCR, qPCR, and cultivation methods in the two areas showed variable prevalence. In Italy, qualitative PCR reported values from 10 to 30% while detection was higher using qPCR values between 30 and 35%. RFTM detection was comparable with the qPCR although always more efficient than PCR from Casas et al. (2002). In Spain, PCR found positive samples in 13.30% of the animals in the two areas.

3.3 Molecular analysis: *Perkinsus* phylogeny and haplotype

The infected animals resulted in positive detection for the genus-specific primers for *Perkinsus* with a 648 bp amplicon. The species identity was checked by sequencing followed by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In samples from Italy, BLAST analysis showed a 96% similarity of *P. olseni* with samples from New Zealand, Australia, and Uruguay and in bivalves such as *Pitar rostrata*, *Astrovenus* sp., and *Haliotis* sp., whereas in Spain, the identity was 99% with samples from South Korea such as *Anadara granosa* (GenBank Accession number: OP961719-OP961722).

The phylogenetic tree resulting from Maximum likelihood is shown in Figure 4. All *Perkinsus* spp. sequences from this study groups together *P. olseni* from Italian and Spanish mussels but places them more distant from other *P. olseni* described in the clams from

TABLE 1 List of the primers used to detect *Perkinsus* spp. with PCR and qPCR in this study.

Primer Name	Sequence	Target species	Reference
PerkITS-85	5′-CCG CTT TGT TTG GAT CCC-3	*Perkinsus* sp.	Casas et al. (2002)
PerkITS-750	5′-ACATCAGGCCTTCTAATG ATG-3	*Perkinsus* sp.	Casas et al. (2002)
Perk-ITS-qF1	5′- CTGACCGCCTTAACGGGC-3′	*P. olseni*	Ríos et al., 2020
PerkITS-qR2	5′- CTATCTCCGAAGAGTTAGTCC-3	*P.olseni*	Ríos et al., 2020

TABLE 2 Survey results of *Perkinsus* infection in the blue mussel, *M. galloprovincialis* collected from Campania region and Ebro Delta. n.p.: analysis not performed.

Location	Sites	Dates	Species	*n*	Shell height (mean ± SD mm)	Prevalence histology	Prevalence PCR	Prevalence qPCR	RFTM
1	Naples, farm	7th February, 2019	*M. galloprovincialis*	20	56.5 ± 6.1	4/20 = **20%**	5/20 = **25%**	6/20 = **30%**	n.p.
2	Naples, farm	21th February 21, 2020	*M. galloprovincialis*	20	28.1 ± 4.2	3/20 = **15%**	6/20 = **30%**	7/20 = **35%**	5/20 = **25%**
3	Naples, farm	27th November, 2020	*M. galloprovincialis*	30	59.4 ± 7.7	6/30 = **20%**	7/30 = **23%**	9/30 = **30%**	8/30 = **26%**
4	Naples, farm	7th April, 2021	*M. galloprovincialis*	30	65.6 ± 8.1	7/30 = **23%**	9/30 = **30%**	9/30 = **30%**	9/30 = **30%**
5	Bagnoli, natural bank	7th May, 2020	*M. galloprovincialis*	30	40.4 ± 4.6	2/30 = **6,6%**	3/30 = **10%**	n.p.	n.p.
6	Ebro Delta A	20th July, 2020	*M. galloprovincialis*	10	61.7 ± 5.9	2/10 = **20%**	2/10 = **20%**	n.p.	n.p.
7	Ebro Delta B	20th July, 2020	*M. galloprovincialis*	10	60.6 ± 6.5	1/10 = **10%**	2/10 = **20%**	n.p.	n.p.

Europe such as clams from Italy, France, and Spain. In Spain, in particular, they are distant from *Perkinsus* sp. such as those from Galicia and from sequences previously described from clams from the Ebro Delta. The genetic distance between these isolates and other isolates of *P. olseni* from different geographic locations ranged from 0.20 to 0.31. The pairwise genetic distance between the *P. olseni* isolates from Campania with those of Spain varied from 0. 31 to 0.27, respectively (Supplementary Table 3).

We found 20 different ITS haplotypes among 163 ITS sequences (Figure 5). One of the biggest haplogroups was composed of haplotypes from Asian countries (Korea, Thailand, and Japan), Europe (Spain, Italy, and France), and few sequences from Brazil. The other haplotypes were divided into the two major haplogroups, which were separated by at least three nucleotide differences. *Perkinsus* sp. in mussels from Aflacs Bay was grouped in another haplogroup containing sequences of bivalves from Europe, New Zealand, Japan, and Korea. The sequences from New Zealand belonged to the New Zealand cockle *Austrovenus stutchburyi*. *P. olseni* in Naples Bay belonged to an isolated haplogroup close to the Aflacs Bay haplogroup.

4 Discussion

This is the first report of *P. olseni* in *Mytilus galloprovincialis* in the Mediterranean Sea. Perkinsosis due to *Perkinsus olseni* in an OIE-listed disease impacts on the health and fitness of populations of many bivalve species (Villalba et al., 2004). Previous works reported the presence of *Perkinsus* species in other mytilidae group, in Japan and Argentina, also describing strong inflammatory lesions connected to the pathogen presence. *P. marinus* and *P. olseni* are the most devastating species and associated with massive mortalities and economic loss. *P. olseni* typically parasitizes Manila clams in Europe and Asia, the carpet shell *R. decussatus* in Europe as well as a the gastropod *Haliotis ruber* in the South of Australia (Lester and Davis, 1981).

Recent advancement of the *in vitro* culture of *Perkinsus* spp. improved the understanding of the biology of the parasite, its pathogenicity and virulence and the impact on the host like clams and abalone (La Peyre, 1993; Soudant et al., 2013; Ruan et al., 2019). On the other side, literature report that mussels could be less affected by infectious agents compared with other bivalves' species (Auguste et al., 2020; Moreira et al., 2020). Because of the scarce knowledge on mussel–*Perkinsus* relationship, it is critical to define the potential negative effect on this new host and consider pathogen prevalence, intensity, and aspects of the inflammatory response. Perkinsosis is a parasitic disease that develops essentially on an inflammatory basis. In clams, the parasite destroys the epithelia and damages the basal membranes of digestive tissues and gills. It is distributed in different tissues and organs, causing an intense inflammation characterized by the formation of infiltrates, nodules, and capsules, to surround and destroy the pathogen, incorporated in an abundant visible PAS-positive substance, a lectin, secreted by the haemocytes (Montes et al., 1995; Montes et al., 1996; Kim et al., 2006; Soudant et al., 2013). In mussels from this study, *P. olseni* showed different tropisms, rarely present in the epithelia, a feature observed in other bivalve species such as the blood cockle *Anadara kagoshimensis* from Korea (Cho et al., 2022) and in other mussel species (Itoh et al., 2019; Vazquez et al., 2022). In the Mediterranean mussel, *Perkinsus* spp. trophozoites were mainly present into haemal spaces in the vesicular connective tissues that surround mussel digestive and reproductive organs, extending into the mantle. Haemocytes, probably granulocytes, perform phagocytosis accompanied by the formation of a capsule to guarantee pathogen elimination. In many cases, recruited granulocytes go through apoptosis, a mechanism that can be part of the mussel immune response or that could be performed by the parasite to evade host immune defence (Soudant et al., 2013). For example, *Perkinsus marinus* was found to modulate apoptosis of eastern oyster haemocytes challenged *in vitro* (Hughes et al., 2010).

Pathogen preference for specific tissues can have an impact on its life cycle and expansion into the host. For example, it may promote its persistence into the tissue, amplify transmission potential, and it can be associated with key virulent phenotypes. *P. olseni* is defined as

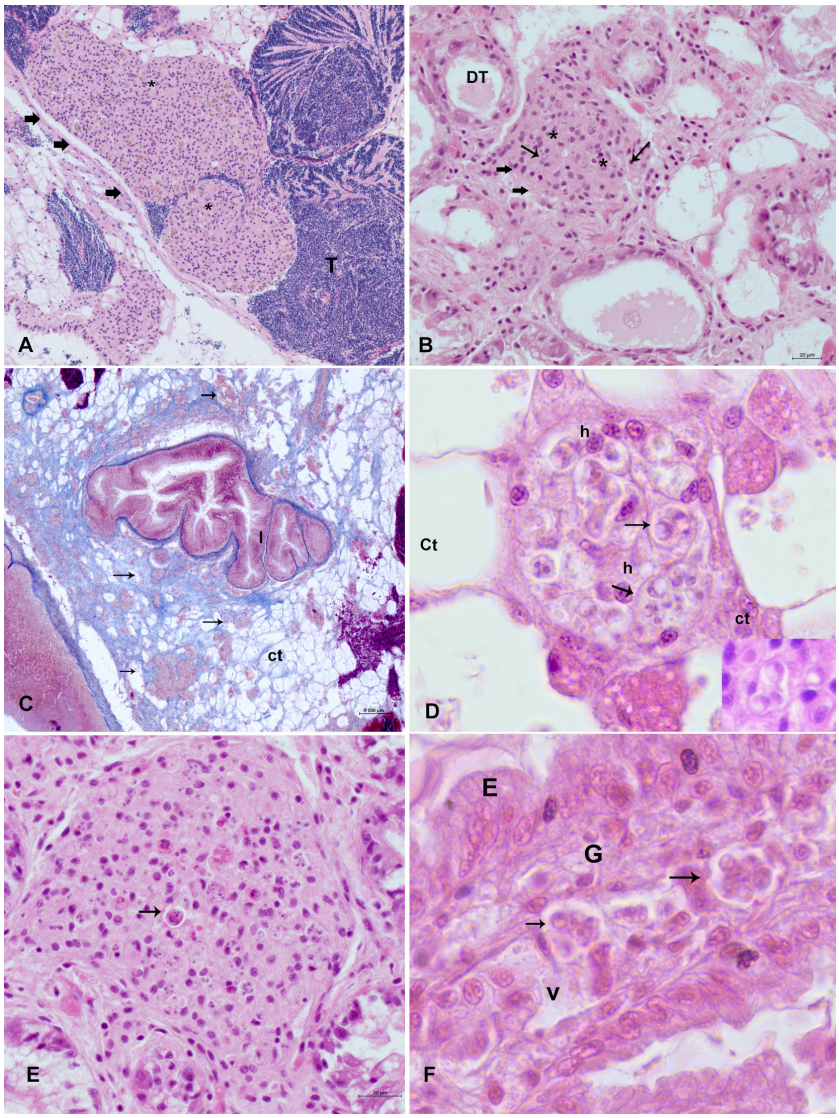

FIGURE 2
Histopathology of mussels affected by Perkinsosis in mussels (*M. galloprovincialis*) in Italy and Spain. **(A, B)** typical feature of the inflammatory lesion (big arrows) with haemocytes (*) related to *Perkinsus* (small arrows): haemocytes nodulation in gonadal follicle **(A)** and the interstitial space of digestive tubules **(B)** big arrows. **(C)** detail of the reactive connective tissue, underlined by Mallory Trichrome in light blue with inflammatory capsules (arrows); **(D)** detail of a capsule in the connective tissue space with haemocyte (h) phagocyting trophozoite of *Perkinsus* (arrow); **(E)** inflammatory capsule displaying apoptotic haemocytes (arrow) with visible trophozoite. **(F)** *Perkinsus* (arrows) in the gill **(G)** haemal vessel in samples from Spain; E, epithelium.

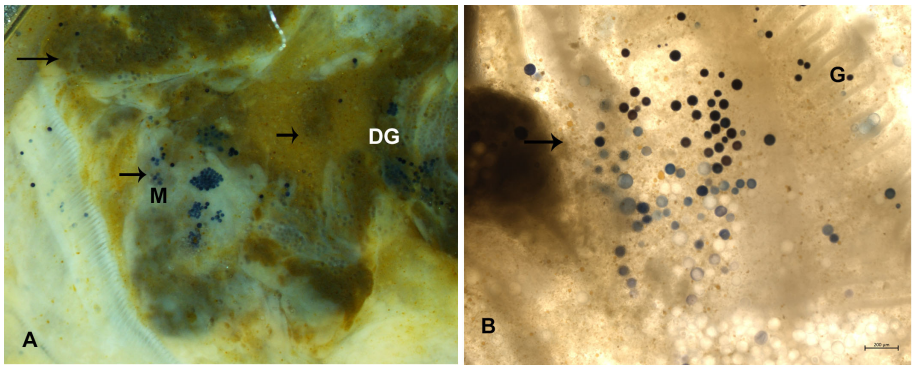

FIGURE 3
Ray's fluid thioglycolate medium (RFTM) assay. **(A)** *Perkinsus* hypnospores in very heavy infection in *M. galloprovincialis* in Campania in digestive tissue (DG), muscle (M) and mantle **(A)** and connective tissue close to the gills (G) **(B)**.

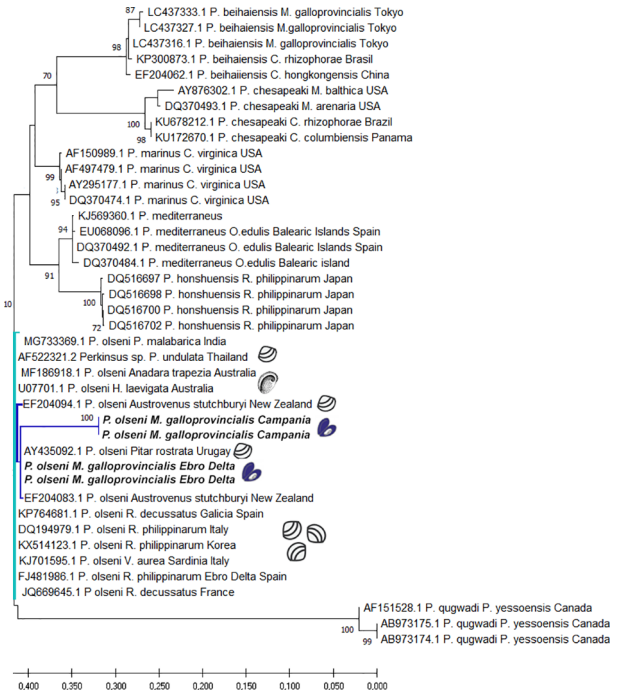

FIGURE 4
Evolutionary analysis by Maximum Likelihood method of *Perkinsus* spp. ITS sequences. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 41 nucleotide sequences. There was a total of 794 positions in the final dataset.

BHR, as other *Perkinus* species such as *P. mediterraneaus*, *P. chesapeaki*, and *P. beihaiensis* presenting a Broad Host Range possibility, similarly to the trend observed for and *Marteilia refringens* that can affect both oysters and mussels (Itoïz et al., 2022; Le Roux et al., 2001; Carella et al., 2010; Carrasco et al., 2012; Arzul et al., 2014; Guo and Ford, 2016). Direct impact of transboundary animal diseases in aquaculture is a significant limitation for living animal's international trade, causing a high socio-economic impact on aquaculture exporting nations. Considering *Perkinsus* plasticity to many bivalve species, like other mollusc's pathogens, recognized international organizations should take this into account in the view of possible cross infection.

The RFTM assay was effective in detecting *Perkinsus* infection, and qPCR was the most sensitive in define pathogen presence. Both methods can be advised to detect a new infection and its prevalence in a given area. The result of phylogeny strongly suggests parasite transfer from clams of Asia and Australia, providing evidence that *P. olseni* from Italian and Spanish mussels are grouped together, but are genetically distant from other *P. olseni* described in Europe (Italy, France, and Spain). Moreover, haplotype network analysis revealed one haplogroup for mussels in Italy and another haplogroup for mussels in Spain, strictly linked to clams from Asia, America, and New Zealand.

The One Health approach acknowledges the connection of human, animal, and ecosystem health. TAADs are highly transmissible, spread very quickly through national borders, causing serious socio-economic consequences. More research is necessary for biosecurity and protection of valuable marine food resources for a growing human population. In the absence of proper surveillance of stocks, the movement of mussels could increase the risk of introduction into other areas and in the local natural population. In Europe, surveillance efforts regarding mollusc diseases is different between Member States and partly depends on the amount and the diversity of the shellfish production. Developing an environmentally friendly and competitive farming practice is a priority objective of the EU to reach high standards in terms of animal/human health and consumer protection. Mussel aquaculture accounts for 15% of the global bivalve production. In Europe, Italy is the second main producer after Spain, with about 64,000 tonnes produced per year and considered one of the largest European markets with an average consumption of 120,000 tonnes per year (FAO Fisheries and

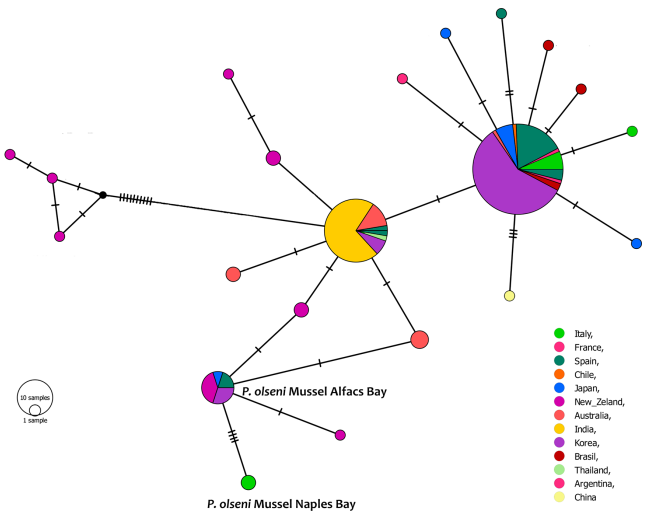

FIGURE 5
TCS network of 163 ITS1 haplotypes of *Perkinsus olseni* estimated by PopART. Each line between haplotypes indicates a single nucleotide substitution. The size of each circle is proportional to the absolute haplotype frequency, **small black circles** represent missing haplotypes, and color shows localities where each haplotype was observed.

Aquaculture Circular, 2020; European Market Observatory for Fisheries and Aquaculture Products (EUMOFA), 2019).

In our study, *P. olseni* prevalence of infection showed a slight pattern of seasonality, as it was higher in warmer seasons than in coldest. Literature reports that this tendency could be due to seasonal seawater temperature changes, as relatively higher temperature during warmer seasons may stimulate *P. olseni* proliferation.

The complex outcome of host–parasite interactions is regulated by different aspects including host biology and immune defence, pathogen virulence, and abiotic factors. Unfortunately, due to the lack of feedback from mussel farmers in Naples Bay, we have no data on possible mortality episodes in the area and how the presence of Perkinsosis could potentially be involved in the fluctuations of the population. Data of mortality are only present from Alfacs Bay, but we cannot conclude that its presence was the trigger of the event. Other studies are needed to define pathogen biology and virulence. *P. olseni* presence in mussels in more than one region of Europe raises possible concerns, considering the high economical value of mussels for the local aquaculture sector in both Italy and Spain.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number : OP961719-OP961722.

Author contributions

Conceptualization: FC. Data curation: FC, MF-T and KA. Formal analysis: FC and GD. Methodology: FC, GV, KA. Writing review and editing; FC, KA, GD, MF-T. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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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.2023.1116837/full#supplementary-material

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Newly detected, virulent *Toxoplasma gondii* COUG strain causing fatal steatitis and toxoplasmosis in southern sea otters (*Enhydra lutris nereis*)

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From February 2020 to March 2022, four southern sea otters (*Enhydra lutris nereis*) stranded in California with severe protozoal steatitis and systemic toxoplasmosis. Three of the infected otters stranded within 26 km of each other, and all four animals died during periods of increased rainfall-driven surface water runoff. High parasite burdens were observed in all tissues except the central nervous system, and toxoplasmosis with severe protozoal steatitis was the primary cause of death for all cases. This lesion pattern differs substantially from all prior reports of toxoplasmosis in sea otters. All cases were *T. gondii*-positive via serology, immunohistochemistry, and PCR. Multilocus sequence typing at 13 loci revealed that all were infected with the same strain of *T. gondii*, previously characterized as an atypical and rare genotype in North America (TgCgCa1, or COUG). The COUG genotype was first isolated from mountain lions in British Columbia, Canada during investigation of a waterborne outbreak of toxoplasmosis in humans. This genotype has not been previously reported from sea otters, nor any aquatic species. All prior *T. gondii* strains obtained from >140 southern sea otters represent Type II or Type X strains, or variants of these genotypes. Archival necropsy data (>1,000 animals over 24 years) were negative for prior cases of severe *T. gondii*-associated steatitis prior to the cases described herein, and no sublethal COUG *T. gondii* infections have been previously indentified in sea otters. According to prior studies, the *T. gondii* COUG genotype is highly virulent in mice and is unusual among *T. gondii* isolates in eliciting a Type I interferon response in murine and human cells *in vitro*; this unusual immunomodulatory response could explain the apparent high virulence of

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this atypical *T. gondii* strain. Our findings reveal a novel and concerning lesion pattern for sea otters with toxoplasmosis. Due to high zoonotic potential and the risk of infection via shared marine food resources, these findings may also indicate potential health threats for other animals and humans.

KEYWORDS

***Enhydra lutris nereis*, genotype, pathology, steatitis, southern sea otter, *Toxoplasma gondii*, toxoplasmosis, virulence**

Introduction

Toxoplasma gondii is a ubiquitous apicomplexan protozoal parasite of significant importance to human and animal health. Wild and domestic felids are the only definitive hosts, and asymptomatic cats can shed millions of environmentally resistant oocysts in their feces (Dubey and Frenkel, 1972; Fritz et al., 2012). Under optimal conditions, sporulated oocysts can remain viable for months (Dumètre and Dardé, 2003), and are infectious to all warm-blooded vertebrates (Tenter et al., 2000). *Toxoplasma gondii* infection can occur via consumption of food or water contaminated with sporulated oocysts, ingestion of tissue cysts in intermediate hosts, and transplacental transmission (Tenter et al., 2000).

In intermediate hosts, *T. gondii* tachyzoites spread systemically, often causing subclinical infection in healthy animals and humans. However, fatal neurological disease can occur, particularly in immunosuppressed individuals. Miscarriage, abortion, and congenital toxoplasmosis are also possible. For individuals surviving initial infection, *T. gondii* tachyzoites respond to the host immune response by converting to bradyzoite-filled tissue cysts in the central nervous system, muscles, and other tissues. If immunity wanes, quiescent tissue cysts can re-activate, again causing systemic disease (Dellacasa-Lindberg et al., 2007; DaSilva et al., 2010; Miller et al., 2018). These diverse transmission strategies and broad host range, coupled with substantial risk to human and animal health, make *T. gondii* one of the world's most globally important pathogens.

Despite being restricted to terrestrial definitive hosts, *T. gondii* can cause morbidity and mortality in diverse marine mammal intermediate hosts, including phocids, otariids, cetaceans, sirenians, and sea otters (Miller et al., 2018). Southern sea otters (*Enhydra lutris nereis*) are especially impacted, with *T. gondii* infecting 62% of sea otters and serving as the primary or contributing cause of death for 8% of otters examined from 1998 through 2012 (Miller et al., 2020). Contributing factors include: 1) Proximity of sea otter habitat to coastal cities, where land-based runoff driven by rainfall can carry freshwater contaminated by oocyst-laden feline feces to nearshore waters, and 2) High consumption of marine bivalves and snails capable of concentrating oocysts from contaminated water (Miller et al., 2008b; Johnson et al., 2009; Krusor et al., 2015).

Although southern sea otters commonly have chronic sublethal *T. gondii* infections, fatal toxoplasmosis also occurs (Shapiro et al., 2016; Miller et al., 2020). Sea otters that have died due to toxoplasmosis usually have no pathognomonic gross lesions other than non-specific lymphadenosplenomegaly; histopathology is required to confirm infection and disease (Miller et al., 2018). Protozoal tissue cysts and occasional tachyzoites are most common in the central nervous system (CNS), accompanied by non-suppurative meningoencephalitis (Miller et al., 2018). For most *T. gondii*-infected sea otters, few parasites are observed microscopically outside of the CNS and occasionally the heart, suggestive of chronic infection (Miller et al., 2018). Death is usually attributed to chronic-active or recrudescent, *T. gondii*-mediated meningoencephalitis and/or myocarditis (Miller et al., 2020).

The four cases described herein exhibited a strikingly different lesion pattern, characterized by grossly apparent, diffuse, severe steatitis affecting all subcutaneous and internal adipose stores. Histopathology revealed severe inflammation and numerous tissue cysts and tachyzoites in most tissues, apart from the central nervous system, where parasite numbers and inflammation were comparatively mild. Multilocus sequence typing revealed that all otters with severe protozoal steatitis were infected with the same atypical *T. gondii* strain, sharing 100% identity across all examined loci. This previously described genotype (COUG or TgCgCa1), was isolated from two mountain lions (*Puma concolor*) during investigation of a large community outbreak of waterborne toxoplasmosis in humans in Canada (Aramini et al., 1999; Dubey et al., 2008; Dubey et al., 2020).

This is the first report of *T. gondii*-associated fatal steatitis and systemic toxoplasmosis in sea otters, and the first report of the COUG *T. gondii* genotype infecting any aquatic animal. The cases described here highlight detection of a previously unreported and virulent strain of *T. gondii* in threatened southern sea otters and underscores the need for detailed studies of *T. gondii* transmission at the land-sea interface.

Materials and methods

Postmortem examination

Stranded southern sea otters were transported to the California Department of Fish and Wildlife's Marine Wildlife Veterinary Care and Research Center in Santa Cruz, CA, where detailed gross necropsies were performed on fresh and frozen-thawed individuals

as previously described (Miller et al., 2020). Sampling methods varied depending on postmortem condition, with formalin fixation of all major tissues for some animals, while others had more limited sampling due to autolysis or freeze-thaw artifact.

Histopathology

Formalin-fixed tissues were embedded, sectioned, and stained with hematoxylin and eosin (H&E) at the Veterinary Medical Teaching Hospital at the University of California, Davis as previously described (Miller et al., 2020). Prepared slides were examined by two veterinary pathologists (MM and DS).

Protozoal immunohistochemistry

Representative paraffin blocks from each case were submitted to the California Animal Health and Food Safety Laboratory at the University of California, Davis for immunohistochemistry using rabbit-derived polyclonal antisera to *T. gondii* and the related apicomplexan parasite *Sarcocystis neurona* using established methods (Miller et al., 2001b). Both parasites are confirmed sea otter pathogens that can appear similar on histopathology, and have similar life cycles, except that the definitive host for *S. neurona* is introduced Virginia opossums (*Didelphis virginiana*) (Miller et al., 2010; Miller et al., 2018). Known-infected tissues were included as positive controls for each parasite, and completed slides were examined by two veterinary pathologists (MM and DS).

Protozoal serology

Where available, postmortem pericardial fluid was assessed for the presence and concentration of *T. gondii* and *S. neurona* IgG antibodies using an indirect fluorescent antibody test (IFAT) as previously described (Miller et al., 2002b), using FITC-conjugated goat anti-ferret IgG antibodies (Bethyl Laboratories Incorporated, Montgomery, Texas). Endpoint titers were determined as the highest dilution that exhibited distinct outline fluorescence of tachyzoites (*T. gondii*) or merozoites (*S. neurona*). Titers ≥ 1:320 were used to establish seropositivity (Miller et al., 2002b).

In vitro parasite cultivation

At necropsy, fresh brain tissue was collected aseptically only from Case 2 to be processed for parasite isolation in cell culture as described (Miller et al., 2001a). Briefly, 5 g of brain tissue was placed in antibiotic/antifungal solution overnight and then homogenized. After homogenization, 1 ml of tissue was incubated in 10 ml trypsin-EDTA (0.25%) at 37°C for 1 hr, added to a feeder layer of MA-104 (monkey kidney) cells, and incubated at 37°C and 5% CO_2 for 2 hr to optimize isolation of *T. gondii*. An additional 1 ml of homogenized tissue was also added directly to a feeder layer of MA-104 (monkey kidney) cells without trypsinization and incubated at 37°C and 5% CO_2 for 2 hr to optimize for isolation of *S. neurona* that are more susceptible to trypsin digestion. After 2 hr, both feeder layers were rinsed with RPMI media supplemented with fetal bovine serum. All cultures were examined thrice weekly for parasite growth and media exchange.

Molecular detection and characterization of protozoal parasites

Nucleic acids were extracted from frozen tissue for all four cases, plus parasite-positive culture supernatant from Case 2, using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Extracted DNA from tissues for each sea otter was initially screened for the presence of *T. gondii* using a nested PCR assay targeting the B1 locus (Grigg and Boothroyd, 2001). Tissue-derived DNA as well as DNA extracted from culture supernatant from Case 2 were further genotyped using a polymerase chain reaction (PCR)-based multilocus sequence typing approach targeting 12 additional polymorphic loci: SAG1, 5'SAG2, 3'SAG2, altSAG2, SAG3, BTUB, GRA6, C22-8, C29-2, L358, PKI, and Apico (Su et al., 2010).

Amplification at the B1 locus was performed as a nested PCR assay using external and internal primers and cycling conditions as described (Grigg and Boothroyd, 2001; Shapiro et al., 2016; Shapiro et al., 2019). The remaining 12 loci were amplified in a multiplex, nested PCR assay using external and internal primers and cycling conditions as previously described (Su et al., 2010). Two loci, C29-2 and Apico, failed to amplify using the multiplex assay and were repeated as simplex, nested PCR assays using the same primers and cycling conditions.

To evaluate for the possibility of co-infection with *S. neurona*, DNA extracted from tissues for Case 4 were additionally screened at the ITS1 locus using previously published primers and cycling conditions (Rejmanek et al., 2010). To identify parasites grown in culture, DNA extracted from trypsinized and untreated culture supernatant was also amplified at the ITS1 locus. To assess for possible co-isolation of *S. neurona*, amplification of the *cox1* locus using *Sarcocystis* spp.-specific primers was performed for both trypsinized and untreated cultures (Gondim et al., 2019).

Products of all PCR assays were sequenced at the UC Davis DNA Sequencing Facility. Sequences were trimmed and aligned to known reference sequences for Type I, Type II, Type III, Type X, and COUG strains at each locus using Geneious software (Biomatters, Auckland, New Zealand). Sequences were further compared to whole genome sequences for reference *T. gondii* strains available in GenBank using BLAST (NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sea otter-derived sequences were deposited in GenBank for all 13 loci characterized in this study and were assigned the following accession numbers: OQ249665 (3'SAG2), OQ249666 (5'SAG2), OQ249667 (altSAG2), OQ249668 (Apico), OQ249669 (B1), OQ249670 (BTUB), OQ249671 (C22-8), OQ249672 (C29-2), OQ249673 (GRA6), OQ249674 (L358), OQ249675 (PKI), OQ249676 (SAG1), OQ249677 (SAG3).

Results

Postmortem examination

From February 2020 through March 2022, four southern sea otters of mixed age and sex (Table 1) presented for necropsy with severe,

TABLE 1 Demographical and geographical data for southern sea otters (*Enhydra lutris nereis*) with fatal *Toxoplasma gondii*-associated steatitis and toxoplasmosis.

Case number	Stranding location	Proximity to next closest case	Stranding date	Condition at stranding	Perimortem clinical signs	Sex/Age class	Nutritional condition
Case 1	San Simeon, San Luis Obispo County	26.5 km	2/18/2020	Alive	Unresponsive, dyspneic, groaning	Female/ Adult	Thin
Case 2	Cayucos East St., San Luis Obispo County	0.5 km	2/8/2022	Fresh dead	Not applicable (found dead)	Female/ Adult	Excellent
Case 3	Natural Bridges, Santa Cruz County	172 km	2/21/2022	Moderately decomposed	Not applicable (found dead)	Male/ Immature	Good
Case 4	Cayucos 4rth St., San Luis Obispo County	0.5 km	3/30/2022	Fresh dead	Not applicable (found dead)	Female/ Adult	Excellent

diffuse, grossly apparent steatitis. Three sea otters stranded within a 26.5 km-span of coastal San Luis Obispo County (two of these animals stranded within 1 km of each other) and one sea otter stranded in Santa Cruz County on the central California coast. The stranding locations for each otter are also shown in a coastal map (Supplementary Figure 1). All four cases died during or after periods of high precipitation in February 2020 (n=1), February 2022 (n=2), and March 2022 (n=1). Only Case 1 had perimortem clinical history (Table 1); all others were found dead. Three otters were sexually mature females, while the other was an immature male. Three animals were classified as good to excellent nutritional condition at necropsy, while one was considered thin, although nutritional condition was difficult to assess in these cases. Three animals were minimally decomposed (one of which had been frozen before necropsy) and one was moderately decomposed and had been frozen before necropsy.

Gross necropsy findings are summarized in Table 2. Putative *T. gondii*-associated gross pathology included severe, diffuse steatitis affecting subcutaneous, visceral, omental, mesenteric, retroperitoneal, and coronary adipose. Affected adipose was diffusely yellow-tan, finely granular to multinodular, and firm to gritty on palpation (Figures 1A, B, 2A–D and Table 2).

Several other tissues exhibited grossly apparent *T. gondii*-associated pathology in addition to systemic adipose (Table 2). Inflamed pancreatic acini were markedly enlarged and firm with scattered intralesional 1-2 cm dark red-black foci (Figures 3A–C). Cardiac involvement was characterized by diffuse orange-white myocardial mottling, epicardial steatitis (Figures 4A, B) and petechia, and viscous pericardial effusion. Lesion overlap between cardiomyopathy associated with domoic acid toxicosis, sarcocystosis, and toxoplasmosis usually precludes discernment between these conditions grossly; histopathology is required (Miller et al., 2020; Miller et al., 2021). However, the severe, grossly apparent epicardial steatitis described herein has not previously been reported from sea otters with fatal toxoplasmosis and may be pathognomonic for this condition. Parasitic pneumonia was characterized grossly by myriad irregular, 2-5 mm diameter, flat, tan-white pleural foci (Figures 5A, B) that were indistinct on cut surface. All lymph nodes were diffusely enlarged, light tan, and firm, with prominent cortical lymphoid hyperplasia on cut surface. Affected spleens were enlarged with prominent lymphoid nodular hyperplasia. In Case 2 the adrenal cortices were congested and hemorrhagic, and copious cloudy, opaque fluid was visible on cut surface especially around the medulla (Figure 6A).

Histopathology

Toxoplasma gondii-associated histopathology findings are summarized in Table 2. Tissues examined microscopically for each case and those positive for *T. gondii* and *S. neurona* are summarized in Supplementary Table 1. Lymphoplasmacytic and variably granulomatous inflammation with numerous intralesional protozoa were observed in many tissues, including subcutaneous and peritoneal adipose, pancreas, brain, heart, lung, liver, lymph nodes, adrenal gland, uterine and intestinal smooth muscle, and skeletal muscle (Table 2). Numerous, approximately 4 x 2 µm tachyzoites and small (e.g., 20-50 µm diameter), thin-walled tissue cysts containing short (4 x 2 µm), stout bradyzoites with eosinophilic cytoplasm were common in areas of severe adipose inflammation (Figures 7A–C). Steatitis was accompanied by multifocal necrosis, edema, hemorrhage, and saponification, with scattered intralesional multinucleated giant cells. Protozoa were especially numerous along the periphery of lipid vacuoles within the cytoplasm of infected adipocytes (Figure 7C). Although adipocytes were often somewhat atrophic on histopathology, severe inflammation resulted in an apparent increase in adipose amount at necropsy, complicating accurate gross assessment of nutritional condition.

In addition to widespread steatitis, several other tissues exhibited severe *T. gondii*-associated lesions (Table 2). Necrotizing pancreatitis and peripancreatic steatitis were associated with numerous intralesional *T. gondii*-like tachyzoites and tissue cysts. Severe myocarditis was also associated with high numbers of intralesional *T. gondii*-like protozoa (Figure 4C). White pulmonary pleural foci corresponded microscopically with regions of lymphoplasmacytic, histiocytic, and necrotizing pneumonia with numerous intralesional tachyzoites and tissue cysts (Figure 5C). Protozoal adrenalitis was characterized by marked inflammation and necrosis with intralesional tachyzoites and tissue cysts (Figures 6B, C), especially in the adrenal medulla, corresponding with the location of cloudy fluid observed grossly. Inflamed lymph nodes exhibited marked paracortical lymphoid hyperplasia with numerous intralesional protozoa. Grossly apparent splenomegaly was characterized microscopically by lymphoid hyperplasia and lympholysis, and

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TABLE 2 *Toxoplasma gondii*-associated gross and microscopic pathology for southern sea otters (*Enhydra lutris nereis*) with fatal *T. gondii*-associated steatitis and toxoplasmosis.

Category	Case 1	Case 2	Case 3	Case 4	Parasite-associated lesion patterns (Gross and microscopic)
Primary cause of death	Systemic toxoplasmosis with steatitis	Systemic toxoplasmosis with steatitis	Systemic toxoplasmosis with steatitis	Systemic toxoplasmosis with steatitis	N/A
Contributing causes of death	Gastric ulcers, melena, emaciation	Probable subacute domoic acid toxicosis	Gastric ulcers, melena, emaciation	Gastric ulcers, melena, emaciation	N/A
Incidental findings	Intestinal parasites (IP)	Congenital renal cyst and IP	None	None	N/A
Carcass condition at necropsy	Euthanized, fresh dead	Fresh dead	Moderately decomposed, frozen-thawed	Fresh dead, frozen-thawed	N/A
Gross necropsy: Relative severity of grossly apparent, putative *T. gondii*-associated pathology					
Subcutaneous and peritoneal steatitis	Severe	Severe	Severe	Severe	Firm, slightly gritty, irregular, yellow to tan-white nodular foci throughout adipose stores
Pancreatitis	Moderate to severe	NE	NE	NE	Pancreatic acini enlarged, firm, and edematous with scattered 1-2 cm dark foci
Myocarditis	Moderate with epicardial petechia and viscous pericardial effusion	Moderate with epicardial steatitis	NE	NE	Orange-white myocardial mottling +/- epicardial petechia, steatitis and viscous effusion
Pneumonitis	Moderate	NE	NE	NE	Thousands of irregular, 2-5 mm diameter flat, tan-white pleural foci
Lymphadenitis	Moderate	Moderate	NE	NE	Affected lymph nodes large, solid, tan, firm
Splenomegaly	NE (Euthanized)	Marked	NE	Moderate	Diffusely enlarged and meaty, prominent lymphoid nodular hyperplasia
Adrenalitis	NE	Severe	NE	NE	Adrenal corticomedullary congestion, hemorrhage, and edema
Histopathology: Relative severity of *T. gondii*-associated histopathology/Suspect parasites observed via microscopy or immunohistochemistry					
Subcutaneous and peritoneal steatitis	Severe/+	Severe/+	Moderate to severe/+	Severe/+	Lymphoplasmacytic, histiocytic and granulomatous steatitis, saponification, fibroplasia, intralesional *T. gondii*-like protozoa
Pancreatitis	Severe/+	Moderate to severe/+	NE	Moderate/+	Necrotizing pancreatitis, peripancreatic steatitis, saponification, *T. gondii*-like protozoa
Myocarditis	Severe/+	Moderate to severe with epicardial steatitis/+	Moderate to severe/+	Moderate to severe/+	Lymphoplasmacytic and histiocytic myocarditis, numerous *T. gondii*-like protozoa
Pneumonitis	Mild to moderate/+	Moderate to severe	NE	Mild to moderate/+	Lymphoplasmacytic and histiocytic pneumonia, *T. gondii*-like protozoa, sparse giant cells
Lymphadenitis	Moderate to severe/+	Moderate to severe/+	NE	Mild to moderate/+	Lymphoplasmacytic and histiocytic lymphadenitis, lymphoid hyperplasia, intralesional *T. gondii*-like protozoa
Adrenalitis	NE	Moderate to severe/+	NE	NE	Severe necrotizing adrenalitis, especially medulla, numerous *T. gondii*-like protozoa
Uterine myometritis	NE	Moderate/+	NA	NE	Lymphoplasmacytic and histiocytic myometritis, myonecrosis and myophagia, sparse intralesional *T. gondii*-like protozoa
Mural enteritis (duodenum)	Moderate/+	NE	NE	NE	*T. gondii*-like protozoa and inflammation in tunica muscularis, most concentrated near parasympathetic ganglia
Skeletal myositis	Mild/+	Mild to moderate/+	NE	Mild/-	Mild multifocal myositis. *T. gondii*-like parasite burden, although low, comparatively high for "classical" toxoplasmosis in sea otter muscle
Meningoencephalitis	Mild to moderate/+ (very few)	Mild to moderate/+	Mild/+ (very few)	Mild/+ (very few)	Mild lymphoplasmacytic and histiocytic nodular meningoencephalitis with sparse intralesional *T. gondii*-like protozoa, when compared with parasite burden in other tissues and previously described sea otter cases

No intralesional ("-") or positive *T. gondii*-like parasites ("+") visible on histopathology and/or immunohistochemistry,
"NE" = Condition not assessed during gross necropsy or histopathology. "N/A" Condition or tissue not applicable due to sex of animal.

marked red pulp plasmacytosis, histiocytosis, and extramedullary hematopoiesis, although parasites were sparse.

The left uterine horn of Case 2 was mildly enlarged grossly (Figure 8A), and mild endometrial hemorrhage, hemosiderosis, and partial atrophy of mural arterioles were observed microscopically, suggestive of recent parturition or abortion. The myometrium of the left uterine horn was severely inflamed, with numerous intralesional *T. gondii*-like tachyzoites and tissue cysts (Figures 8B, C); the endo- and mesometrium exhibited milder inflammation and fewer parasites. The right uterine horn was minimally inflamed, and few parasites were observed microscopically. Moderate inflammation and *T. gondii*-like tachyzoites and tissue cysts were also observed in the duodenal tunica muscularis in Case 1, with inflammation and parasites most concentrated near parasympathetic ganglia.

In contrast with widespread inflammation and high parasite burdens observed in other tissues, brains of affected otters exhibited surprisingly mild meningoencephalitis with sparse intralesional tachyzoites and tissue cysts, unlike typical patterns reported from sea otters with fatal toxoplasmosis (Miller et al., 2018; Miller et al., 2020). Throughout all examined tissues, tissue cysts and tachyzoites were most common on histopathology in the cytoplasm of adipocytes, pancreatic acinar cells, and smooth, skeletal, and cardiac myofibers. Protozoa were also observed within the cytoplasm of fibroblasts, macrophages, monocytes, lymphocytes, hepatocytes, adrenal cortical and chromaffin cells, and cerebral neurons and glia.

Protozoal immunohistochemistry and serology

Results of *T. gondii*-associated immunohistochemistry are summarized in Table 2, and composite results of protozoal histology, immunohistochemistry, and serology for *T. gondii* and *S. neurona* are summarized in Supplementary Table 1. Formalin-fixed tissues from all four cases contained tachyzoites and tissue cysts that were strongly immunopositive for *T. gondii*. Inflamed subcutaneous and peritoneal adipose (Figures 7D, E), pancreas (Figure 3D), heart (Figure 4D), lymph nodes, and brain (Figure 6D) were *T. gondii*-immunopositive for all four otters (Table 2). Lung (Figure 5D) and skeletal muscle were *T. gondii* immunopositive for two of three cases where this tissue was available for microscopic examination. The inflamed adrenal gland and uterine myometrium (Figure 8D) from Case 2 were also strongly *T. gondii*-immunopositive.

Immunohistochemistry for *S. neurona* was performed on the same tissues as for *T. gondii*, and protozoa in all tissues from Cases 1, 2, and 3 were diffusely immunonegative (Figure 6E). Case 4 exhibited sparse *S. neurona*-positive staining in myocardium, and potential small foci of positive staining in subcutaneous adipose and lung. Pericardial fluid was available for Cases 1, 2, and 4; all had high positive *T. gondii* IgG (≥1:10,240), and low positive *S. neurona* titers (≤1:2,560) (Supplementary Table 1).

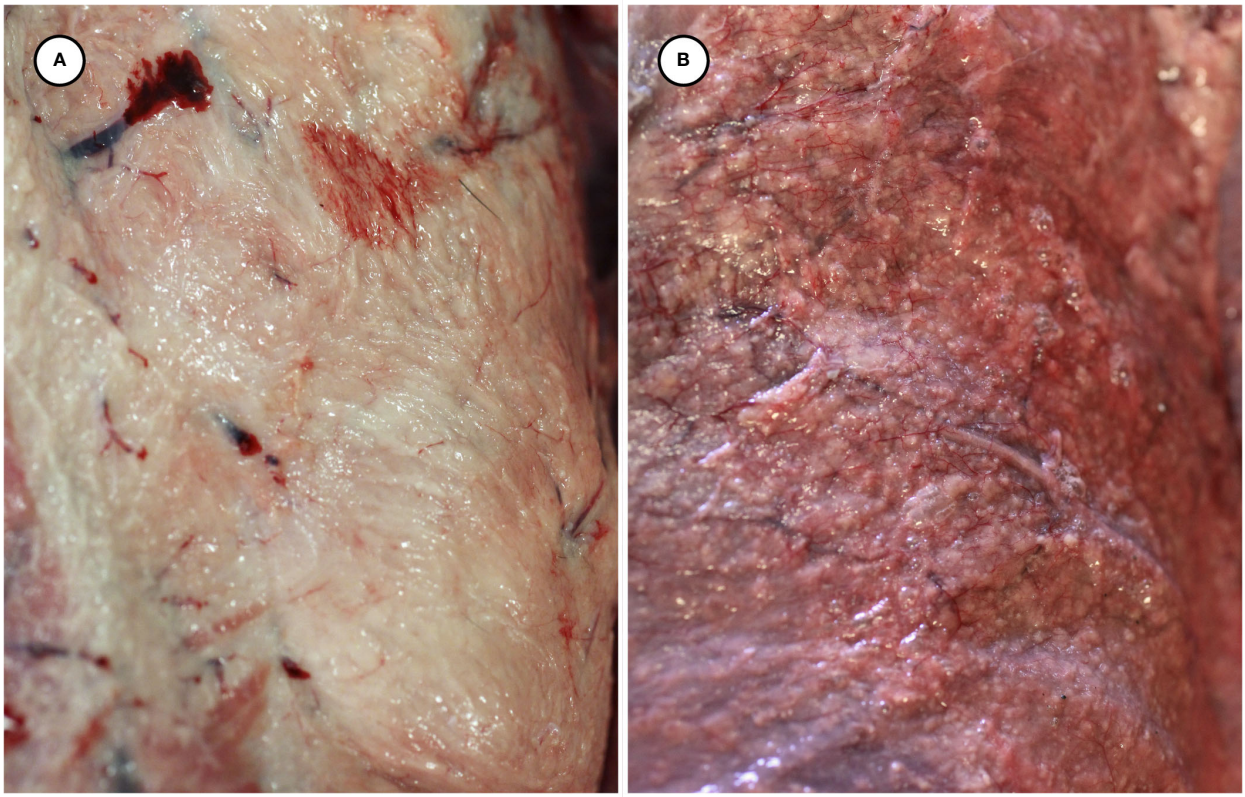

FIGURE 1
Gross appearance of normal subcutaneous adipose from an unaffected sea otter **(A)**, compared with congested and multinodular-appearing adipose from a sea otter with fatal protozoal steatitis and toxoplasmosis **(B)**.

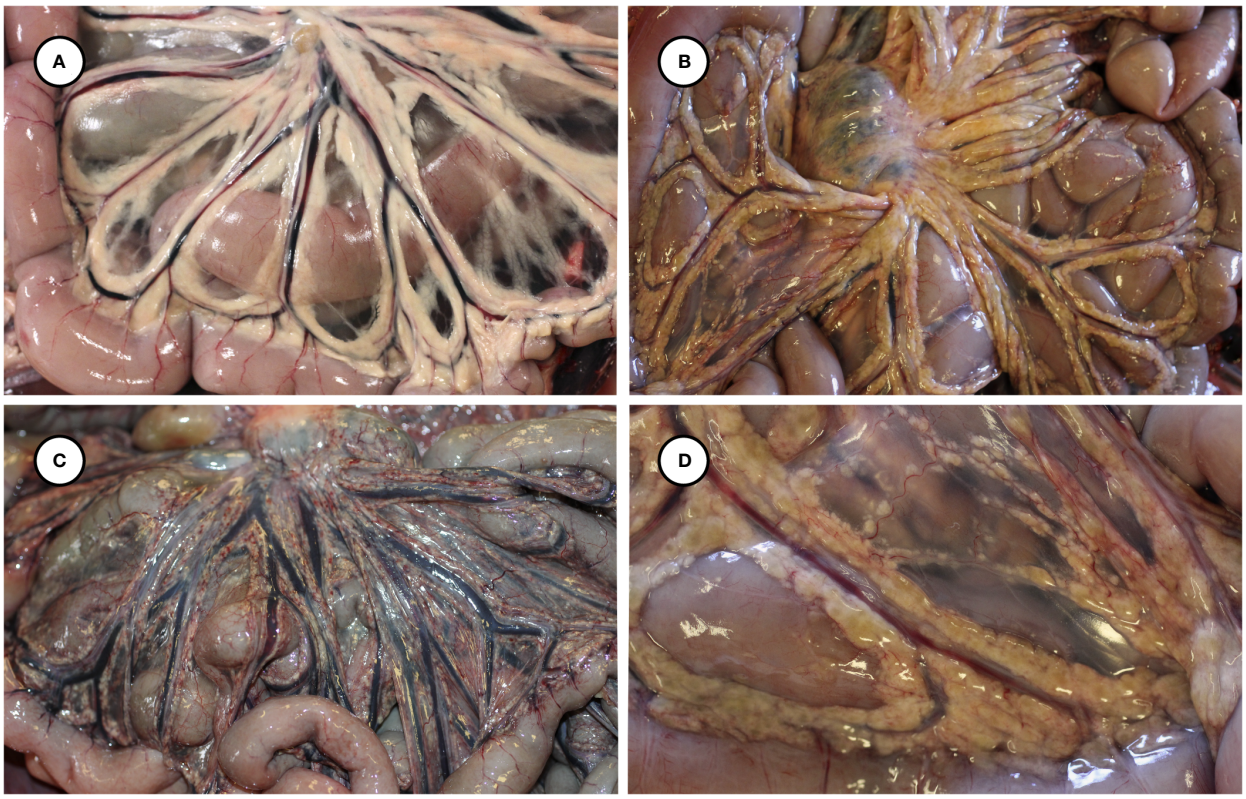

FIGURE 2
Gross appearance of normal mesenteric adipose from an unaffected sea otter **(A)**, compared with yellow-discolored, variably congested, multinodular-appearing mesenteric adipose at low **(B, C)** and higher magnification **(D)** in sea otters with fatal protozoal steatitis and toxoplasmosis.

In vitro parasite cultivation

Parasites morphologically resembling *T. gondii* and *S. neurona* were observed in trypsinized and untreated cell cultures from Case 2 within two weeks of inoculating cells with brain tissue. Amplification and sequencing of DNA extracted from supernatant at the ITS1 locus yielded 100% identity to *T. gondii* in both trypsinized and untreated cultures. Amplification and sequencing of the *cox1* locus yielded 100% identity to *S. neurona* in supernatant from the untreated culture, while DNA amplification at the *cox1* locus was not observed for supernatant from the trypsinized culture. These results confirm isolation of *T. gondii* in the trypsinized and untreated cell cultures, and additional co-isolation of *S. neurona* in the untreated culture.

Molecular detection and characterization of protozoal parasites

Results of molecular detection and characterization of protozoal parasites are summarized in Table 3. Genotyping across 12 to 13 loci was successfully performed using DNA from subcutaneous adipose from all four sea otters, plus culture supernatant from inoculated brain tissue of Case 2. Initial local sequence alignment revealed that the *T. gondii* strains from all animals shared 100% sequence identity with each other across all examined loci (Supplementary Figure 2), plus several polymorphisms when compared to known reference sequences for Type I, Type II, Type III, and Type X strains. Subsequent analysis compared the identity of the sea otter *T. gondii* sequences to whole genome sequences from publicly available *T. gondii* reference genotypes on GenBank. This analysis revealed 100% identity across the 13 examined loci of *T. gondii* sequences from all four otters to the COUG strain (TgCgCa1) (Supplementary Figure 2).

Final diagnoses

Based on cumulative findings from gross necropsy, histopathology, immunohistochemistry, and molecular parasite confirmation, the primary cause of death was systemic toxoplasmosis with severe steatitis for all four cases (Table 2). Given their relatively good nutritional condition, high systemic parasite load, and relative paucity of parasites and parasite-associated inflammation in the CNS, affected animals appear to have died in the acute or subacute stages of *T. gondii* infection (Miller et al., 2018).

Contributing causes of death and incidental findings are summarized in Table 2. Methods used for lesion assessment and ranking were as previously described (Miller et al., 2020; Miller et al., 2021). Findings suggestive of subacute to chronic domoic acid toxicosis (Miller et al., 2021) in Case 2 were characterized by moderate, diffuse brain congestion; segmental cytoplasmic

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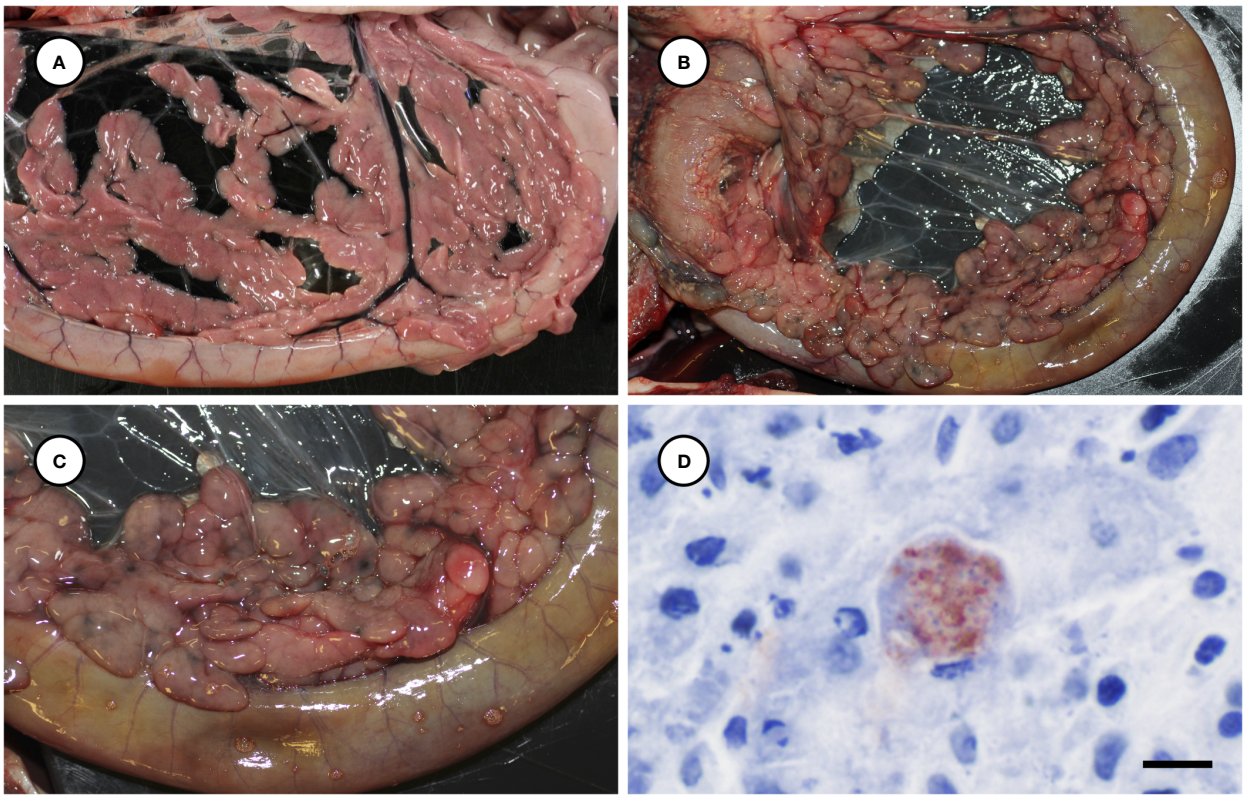

FIGURE 3
Normal pancreas from an unaffected sea otter **(A)**, compared with low **(B)** and higher magnification **(C)** views of swollen, edematous, red-black discolored pancreas from a sea otter with protozoal pancreatitis. Immunohistochemical stain of inflamed pancreas demonstrating strong positive immunostaining of intralesional *T. gondii* parasites (**D**: Bar = 10 µm).

vacuolation and angularity of hippocampal pyramidal neurons; CA2 sector neuronal depletion; and a mildly shrunken hippocampal profile. Temporally concordant cardiac lesions presumed to be associated with domoic acid toxicosis included patchy myofiber loss, stromal collapse, mural fibrosis, and hyalinization of the tunica media of coronary arterioles. Other contributing findings (gastric ulcers, melena, and emaciation) are common in sick or stressed sea otters (Miller et al., 2020).

Cystic degeneration of the caudal pole of the right kidney was considered an incidental finding for Case 2. The cystic structure was filled with pale yellow urine and sediment and was not patent to the ureter. Histopathology revealed diffuse parenchymal atrophy and mild lymphoplasmacytic inflammation. The inner cyst wall was lined by a single layer of flattened epithelium overlying a dense fibrovascular stroma. No protozoal parasites were observed within the cyst wall, nor in any histological sections of kidney.

Discussion

This study describes an unusual and severe presentation of toxoplasmosis and steatitis in sea otters infected with a rare *T. gondii* strain not previously reported in any aquatic animal. Observed lesion and case presentation patterns differed substantially from prior descriptions of toxoplasmosis in sea otters, where cases of acute to subacute, severe, disseminated toxoplasmosis were reported mainly from fetuses and neonates (Miller et al., 2008a; Shapiro et al., 2016). In contrast with the cases described herein, sea otters with fatal toxoplasmosis usually have no gross lesions other than non-specific lymphadenosplenomegaly, and histopathology is required to confirm infection (Miller et al., 2018). Protozoa are typically numerous in the CNS, accompanied by non-suppurative meningoencephalitis, but are relatively rare in other tissues on conventional histopathology (Miller et al., 2018). The most common extra-CNS lesion reported from *T. gondii*-infected sea otters is protozoal myocarditis, and death is usually attributed to chronic, active meningoencephalitis or myocarditis (Miller et al., 2020). Although microscopic foci of *T. gondii*-associated steatitis, myositis, pancreatitis, and adrenalitis are occasionally observed, this finding is consistently mild in comparison to the brain and cardiac pathology.

The four cases described herein exhibit a strikingly different lesion pattern, characterized by grossly apparent, diffuse, severe steatitis of all subcutaneous and internal adipose stores.

Affected adipose was yellow-mottled and firm with a nodular appearance. Some cases also exhibited grossly visible pancreatic, pulmonary, adrenal, and cardiac pathology. Histopathology revealed severe inflammation with numerous intralesional protozoa in most tissues, apart from the CNS, where parasite numbers and inflammation were comparatively mild. Severe toxoplasmosis and protozoal steatitis was identified as the primary cause of death in all cases and inflamed tissues were strongly immunopositive, confirming the association of systemic inflammation with *T. gondii* infection.

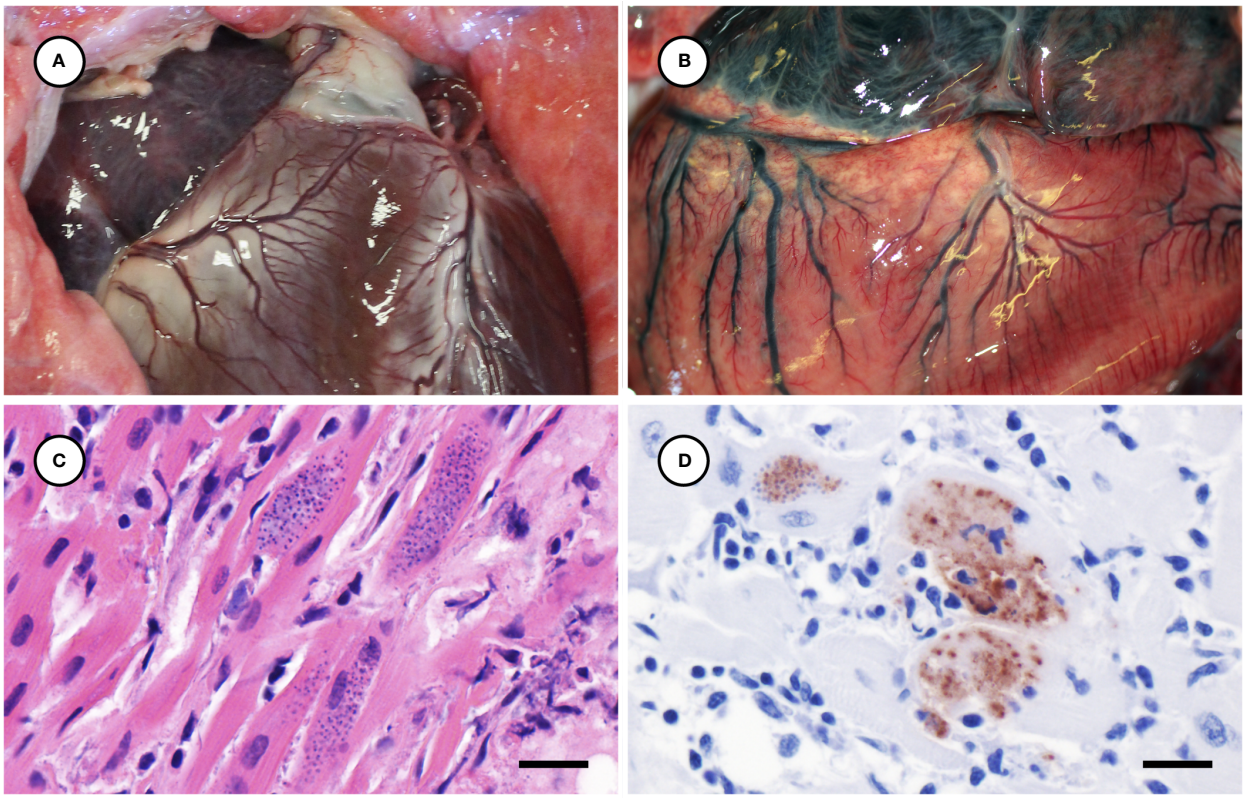

FIGURE 4
Normal heart from an unaffected sea otter **(A)**, compared with orange-discolored myocardium and pale yellow, multinodular-appearing epicardial adipose from a sea otter with protozoal myocarditis and epicardial steatitis **(B)**. Microscopic view of severely inflamed myocardium **(C)** with numerous intralesional *Toxoplasma gondii*-like tissue cysts (H&E stain, bar = 10 µm). Immunohistochemical stain of inflamed myocardium **(D)** demonstrating strong positive immunostaining of intralesional protozoa for *T. gondii* (Bar = 10 µm).

These unusual cases also differed from prior reports of fatal toxoplasmosis in sea otters in other ways: In previous studies (Kreuder et al., 2003: Miller et al., 2020), sea otter deaths due to toxoplasmosis were relatively sporadic, with little variation by season or month. In contrast, all otters with fatal toxoplasmosis with steatitis stranded in February and March, which corresponds with the peak rainy season in California when land-sea runoff following rainfall events is highest. Wet season protozoal disease outbreaks have also been reported for *S. neurona* (Miller et al., 2010; Shapiro et al., 2012), and seasonal land-sea transport of infectious oocysts or sporocysts is considered the most likely route of sea otter infection by both parasites (Miller et al., 2002a; Miller et al., 2008b; Miller et al., 2010).

Affected otters were strongly seropositive for *T. gondii* IgG (≥1:10,240) and multilocus sequence typing at 13 loci revealed that all animals with this unusual lesion pattern were infected with the same atypical COUG strain of *T. gondii* (TgCgCa1). Despite decades of studies on the molecular epidemiology of *T. gondii* in coastal California, including genetic characterization of >140 *T. gondii* isolates obtained from southern sea otters (Miller et al., 2004; Sundar et al., 2008; VanWormer et al., 2014; Shapiro et al., 2015; Shapiro et al., 2016; Shapiro et al., 2019), this is the first report of the COUG strain in sea otters, or any aquatic species. To ensure that prior protozoal steatitis cases had not been missed, archival necropsy data (>1,000 sea otters from 1997 through 2022) from the California Department of Fish and Wildlife was reviewed for additional cases of diffuse, severe *T. gondii*-associated steatitis. None were found other than the current four cases, the earliest of which occurred in 2020.

Importantly, the COUG *T. gondii* strain appears to be capable of causing rapid mortality in ostensibly healthy, prime-aged adult sea otters. Three of the four cases were adult females, while one was an immature male. Possible spatial clustering of cases was also noted within a 26.5 km coastal region in San Luis Obispo County. However, given our small sample size, effects of sex, age, and stranding location cannot be statistically evaluated.

The COUG strain (ToxoDB PCR-RFLP genotype #66) is considered a rare, atypical North American strain circulating in wildlife. Phylogenetically, this strain clusters within the same major clade (D) as the archetypal Type II and atypical Type X strain, but within a distinctly characterized haplotype (11) that it shares with another atypical strain derived from a jaguar (GUY-2004-JAG1) (Lorenzi et al., 2016). The COUG strain was originally isolated via mouse bioassay from fecal samples from two mountain lions collected following epidemiological studies of a large waterborne toxoplasmosis outbreak in humans in British Columbia, Canada that occurred in 1995 (Dubey et al., 2008). One sample was found in the environment, while the second was collected per rectum from a mountain lion that was euthanized in the vicinity of the drinking water reservoir implicated in the outbreak; no information was reported regarding *T. gondii*-associated pathology in the euthanized mountain lion (Dubey et al., 2008). Fecal shedding of oocysts by mountain lions

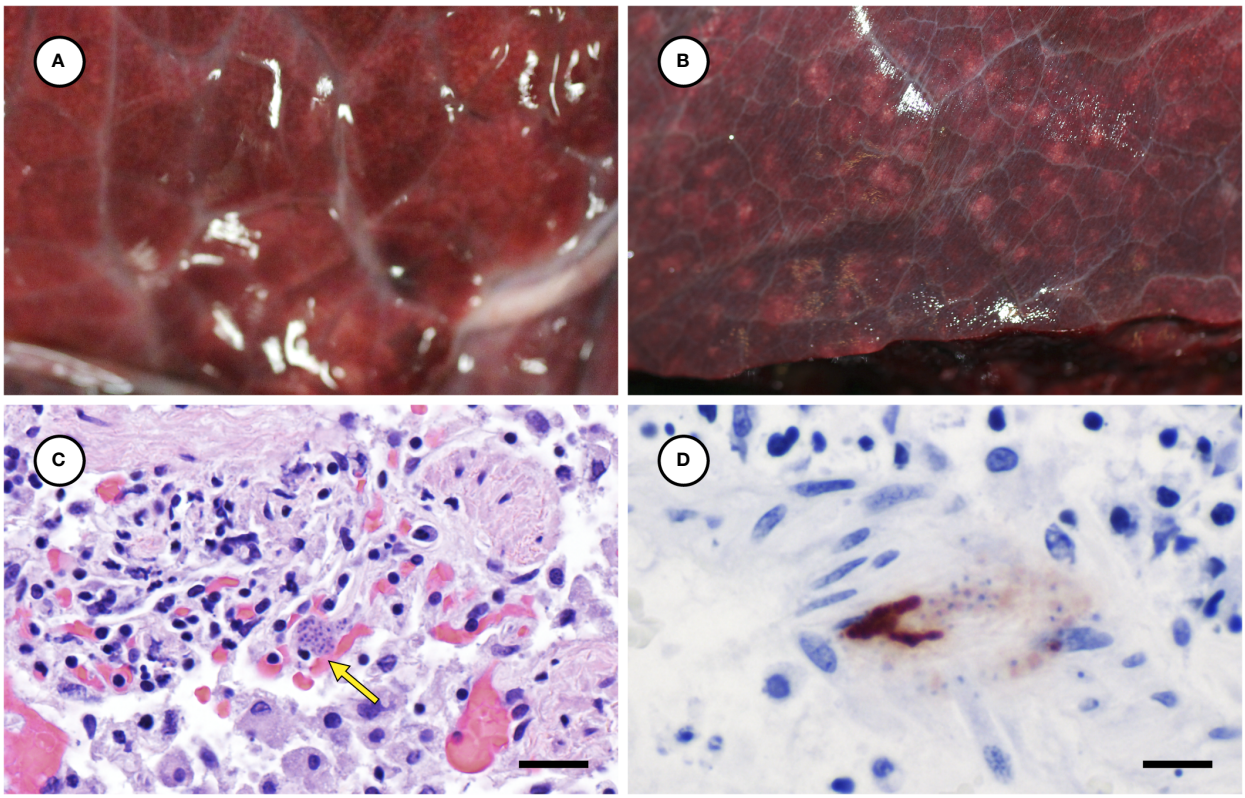

FIGURE 5
Appearance of normal lung from an unaffected sea otter **(A)**, compared to lung from a sea otter with protozoal pneumonia **(B)** containing multiple pale irregular pleural foci. Microscopic view of inflamed lung containing intralesional *Toxoplasma gondii*-like tissue cyst (**C**: arrow) (H&E stain, bar = 20 µm). Immunohistochemical stain of lung demonstrating strong positive immunostaining of intralesional protozoa for *T. gondii* **(D)** (Bar = 10 µm).

or other wild felids was postulated to have served as the source of the toxoplasmosis outbreak (Bowie et al., 1997; Aramini et al., 1999). However, because no information was published on genotype(s) of *T. gondii* in infected humans from the outbreak, there is insufficient evidence linking the COUG strain in sympatric felids to the human waterborne toxoplasmosis event. To our knowledge, the only other animal reported to be infected with a COUG genotype is a feral pig (*Sus scrofa*) from the Sierra Nevada Mountains in eastern California (Dubey et al., 2020).

Although this unique parasite strain could have been present before 2020, no prior molecular studies of *T. gondii* infection in sea otters, domestic cats, mountain lions, bobcats, canids, and other animals along the California coast identified any animals infected with the COUG *T. gondii* strain (Miller et al., 2008b; VanWormer et al., 2013; VanWormer et al., 2014; Shapiro et al., 2019). This knowledge, plus detection of a novel *T. gondii*-associated lesion pattern with high virulence that coincides temporally with detection of COUG *T. gondii* infection in stranded sea otters suggests that introduction of this highly pathogenic strain into coastal California wildlife occurred recently.

Geographical movement of *T. gondii* strains may occur via infected migratory birds, migration of terrestrial wildlife, or anthropogenic translocation of wild animals, livestock, and pets, including domestic cats (Galal et al., 2022). Though isolated previously from wild felids, domestic cats have been shown to contribute substantially more oocysts in coastal California watersheds when compared to bobcats and mountain lions (VanWormer et al., 2013), and cats could be exposed to this wildlife-adapted strain by feeding on wild prey.

It is also unclear at present whether multiple point sources of the COUG *T. gondii* strain may be spreading into the marine environment along the California coast. Multiple sources of exposure could explain the two geographically separate regions where the four cases described in this study were found. Another possibility is that the immature male was initially infected in the same region as the females but stranded elsewhere due to migration post-infection. Previous studies of tagged sea otters revealed that males may travel hundreds of km (Tinker et al., 2017), with immature males frequently dispersing beyond study areas for extended periods (J. Tomoleoni, pers. comm), suggesting that the immature male could have stranded in a different county than the original location of *T. gondii* infection. Interestingly, the three adult females stranded near Morro Bay, a confirmed, high-risk area for *T. gondii* Type X infection and mortality in sea otters (Shapiro et al., 2019). Numerous factors appear to contribute to these high-risk zones, including local geography, precipitation, climate, felid density, and other factors (VanWormer et al., 2013; VanWormer et al., 2014; Burgess et al., 2018; Miller et al., 2020). Given the apparent high virulence and unknown source of this atypical strain, as well as the potential health risks for threatened sea otters, other animals and humans, additional

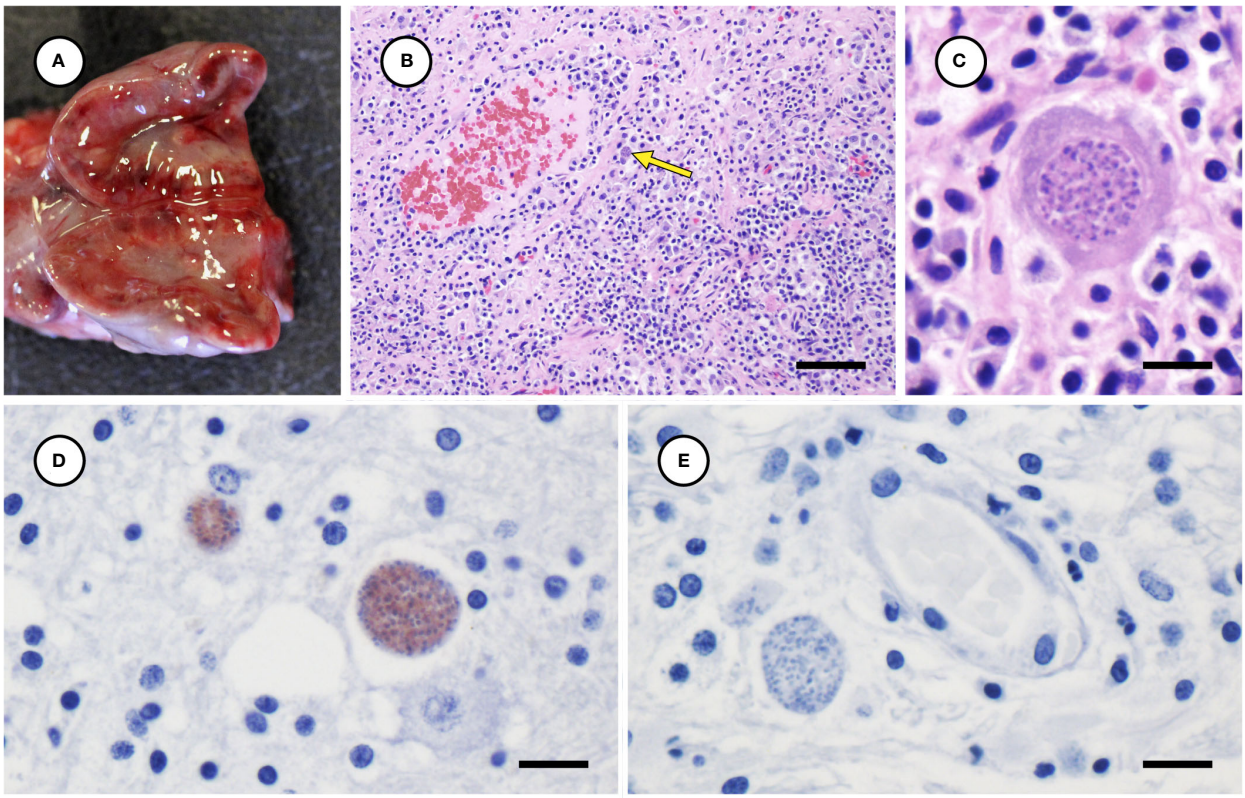

FIGURE 6
Congested, hemorrhagic and edematous adrenal gland from a sea otter with fatal toxoplasmosis and steatitis **(A)**. Low **(B)** and high **(C)** magnification microscopic views of markedly inflamed adrenal medulla from the same sea otter demonstrating intralesional *Toxoplasma gondii*-like protozoa (indicated by arrow for B) (H&E stain, bar = 40 and 10 µm, respectively). Immunohistochemical stains of mildly inflamed brain tissue from sea otters with fatal toxoplasmosis and steatitis, demonstrating relatively mild inflammation and strong positive immunostaining of intralesional tissue cysts for *T. gondii* **(D)**, and negative immunostaining of brain parasites for *Sarcocystis neurona* **(E)** (**D**, **E**: bar = 10 and 10 µm, respectively).

research is needed to clarify the host and geographical distribution of the COUG strain in North America.

This is the first report of severe steatitis associated with toxoplasmosis in sea otters, but similar lesions were reported in Hawaiian monk seals (*Neomonachus schauinslandi*) with fatal toxoplasmosis (Barbieri et al., 2016). Although the monk seal steatitis lesions appear similar grossly and microscopically, the *T. gondii* genotype(s) associated with monk seal infection were not reported. Protozoal steatitis was also reported in a red kangaroo and aborted goats with fatal toxoplasmosis (Chen and Alley, 1987; Dubey and Hartley, 1992).

Although *T. gondii* has been traditionally classified into three archetypal genotypes (Type I, II, and III), more advanced genotyping approaches and global characterization of parasite strains have contributed to much broader recognition of non-archetypal or "atypical" genotypes. Associations between infection by specific *T. gondii* genotypes and more severe disease have been reported in animals and people (Grigg et al., 2001; Khan et al., 2009; Melo et al., 2013). The relationship between strain genotype and virulence is well established in mouse models, with Type I isolates and most South American strains being highly virulent, whereas Type II and III strains are less virulent (Khan et al., 2009; Melo et al., 2013).

The atypical Type X strains (ToxoDB#5) have been classified within haplotype 12 and are predominantly found in North American wildlife (Dubey et al., 2011; Jiang et al., 2018). Southern sea otters are most frequently infected by Type X or X variant strains, which are more pathogenic in sea otters than Type II strains (Miller et al., 2004; Shapiro et al., 2019). In a study of 135 necropsied southern sea otters, 79% of *T. gondii* isolates were Type X or Type X variants, while the remaining 21% were Type II or Type II/X recombinants. Additionally, all enrolled otters with *T. gondii* identified as the primary cause of death were infected with Type X or Type X variants (Shapiro et al., 2019). In contrast, a study of mixed marine mammal species in the Pacific Northwest, including five northern sea otters (*E. l. kenyoni*) revealed no significant associations between *T. gondii* genotype and disease outcome (Gibson et al., 2011).

Although no assessment of associated pathology or disease outcome was included in descriptions of the COUG *T. gondii* strain from mountain lions and a wild pig (Dubey et al., 2008; Dubey et al., 2020), the COUG genotype has been shown to be highly virulent in mice and significantly more virulent than Type II strains (Khan et al., 2009). However, it was unclear if this enhanced virulence was due to a high parasite burden or a strong, parasite-associated inflammatory response. Substantial genetic differences between COUG and Type II *T. gondii* strains have also been reported (Minot et al., 2012).

Another study revealed that the COUG strain is unusual among *T. gondii* isolates in eliciting a Type I interferon response in both murine macrophages and human fibroblasts (Melo et al., 2013). Type I

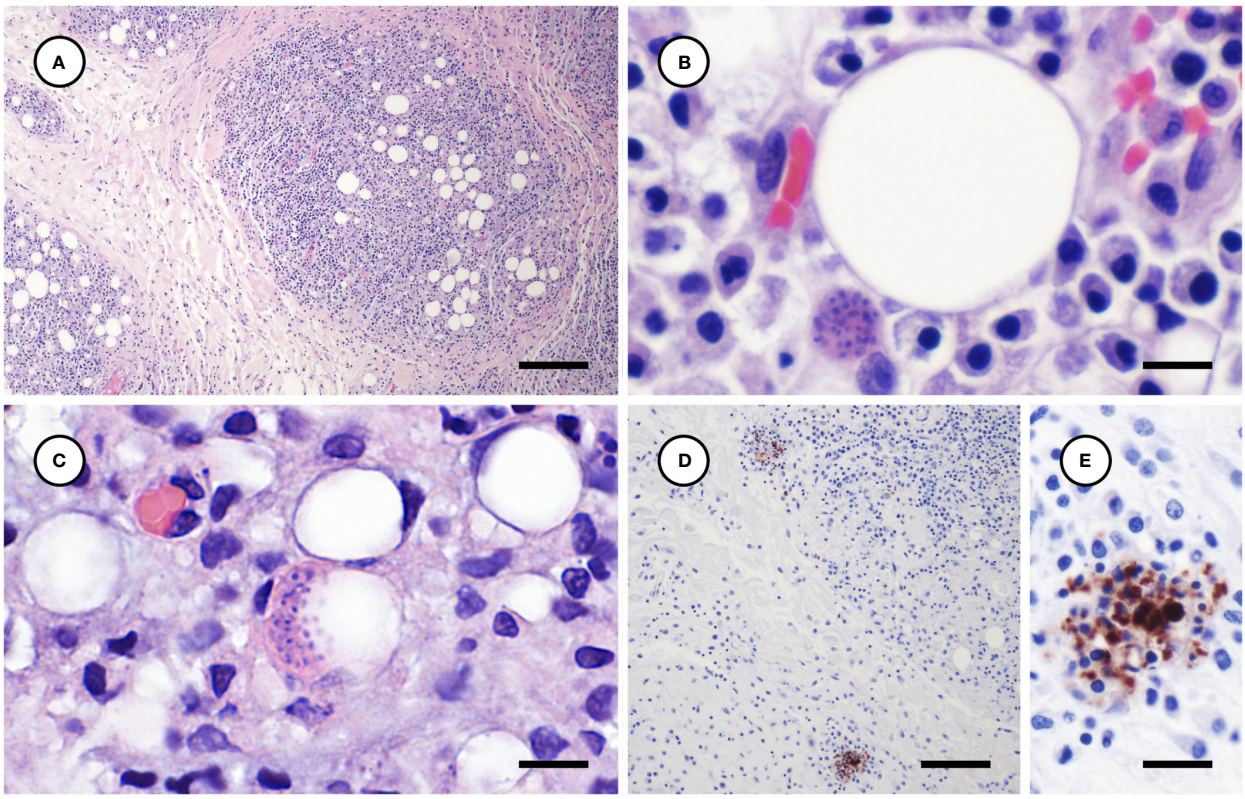

FIGURE 7
Histological and immunohistochemical appearance of subcutaneous and peritoneal steatitis from sea otters with fatal protozoal steatitis and toxoplasmosis. **(A–C)**: Progressively higher magnification views of severely inflamed adipose tissue **(A)**; a *Toxoplasma gondii*-like protozoal tissue cyst is located adjacent to a large lipid vacuole **(B)** and numerous parasites are clustered along the periphery of a cytoplasmic lipid vacuole **(C)** (H&E stain, bar = 200, 10, and 10 µm, respectively). Immunohistochemical stains of inflamed peritoneal adipose, demonstrating strong positive *T. gondii* immunostaining of intralesional protozoa at lower **(D)** and higher **(E)** magnification (**D**, **E**: bar = 100 and 20 µm, respectively).

interferons (IFN), including IFN-α and -β, are immunomodulatory cytokines that serve as potent coordinators of antimicrobial responses. In addition to their well-recognized role in viral immunity, IFNs can also play important roles during parasitic infection by controlling parasite growth through activation of intracellular killing mechanisms or enhancing disease severity. According to Melo et al. (2013), recent reports that Type I IFNs suppress Type II IFN-triggered human anti-mycobacterial responses (Teles et al., 2013) could suggest that the Type I IFN response induced by COUG and other select atypical *T. gondii* strains could be associated with enhanced pathogenicity. In addition, Type I IFNs can affect host cellular lipid metabolism by inhibiting *de novo* cholesterol and fatty-acid synthesis and upregulating the uptake of exogenous lipids (Wu et al., 2016). Although Type I IFNs can induce metabolic changes that are critical for immune function (Wu et al., 2016), these changes could also provide selective advantages for highly lipophilic intracellular protozoa.

The striking tropism of the COUG strain of *T. gondii* for sea otter adipose tissue is unusual and noteworthy. Not only were parasites numerous throughout systemic adipose stores in association with severe inflammation, but on histopathology numerous parasites were concentrated along the periphery of lipid droplets within the cytoplasm of infected adipocytes (Figure 7C). Although *T. gondii* is known to scavenge host lipids to facilitate parasite replication and survival (Nolan et al., 2017), and host-parasite competition for lipid stores likely contributes to disease pathogenesis (Coppens, 2006), the degree of parasitism within adipose tissue in these cases was extreme when compared with hundreds of previous histologically and molecularly confirmed cases of toxoplasmosis in sea otters.

In addition to the grossly apparent steatitis and pancreatitis reported herein, another fascinating and potentially strain-associated difference for COUG *T. gondii*-infected sea otters is significant protozoal infection of smooth muscle cells within the uterine myometrium (Figures 8B–D) and duodenal tunica muscularis (Table 2). Despite screening sea otter tissues for 24 years, one author (MM) has never observed this previously. Although *T. gondii* can infect virtually any nucleated cell type in warm-blooded animals and humans, infection of skeletal and cardiac muscle is most commonly reported, and skeletal muscle appears to be a preferred tissue for persistent *T. gondii* infection (Swierzy et al., 2014). The parasite load and inflammation in the uterine myometrium was particularly severe and could have been associated with fetal infection and abortion, although this was impossible to confirm at gross necropsy. Interestingly, uterine and gastric mural infection was also reported in Hawaiian Monk seals with fatal toxoplasmosis and protozoal steatitis (Barbieri et al., 2016), although the infecting genotype in the described Monk Seal cases have not been reported to date. *Toxoplasma gondii*-associated smooth muscle necrosis has also been reported in highly susceptible

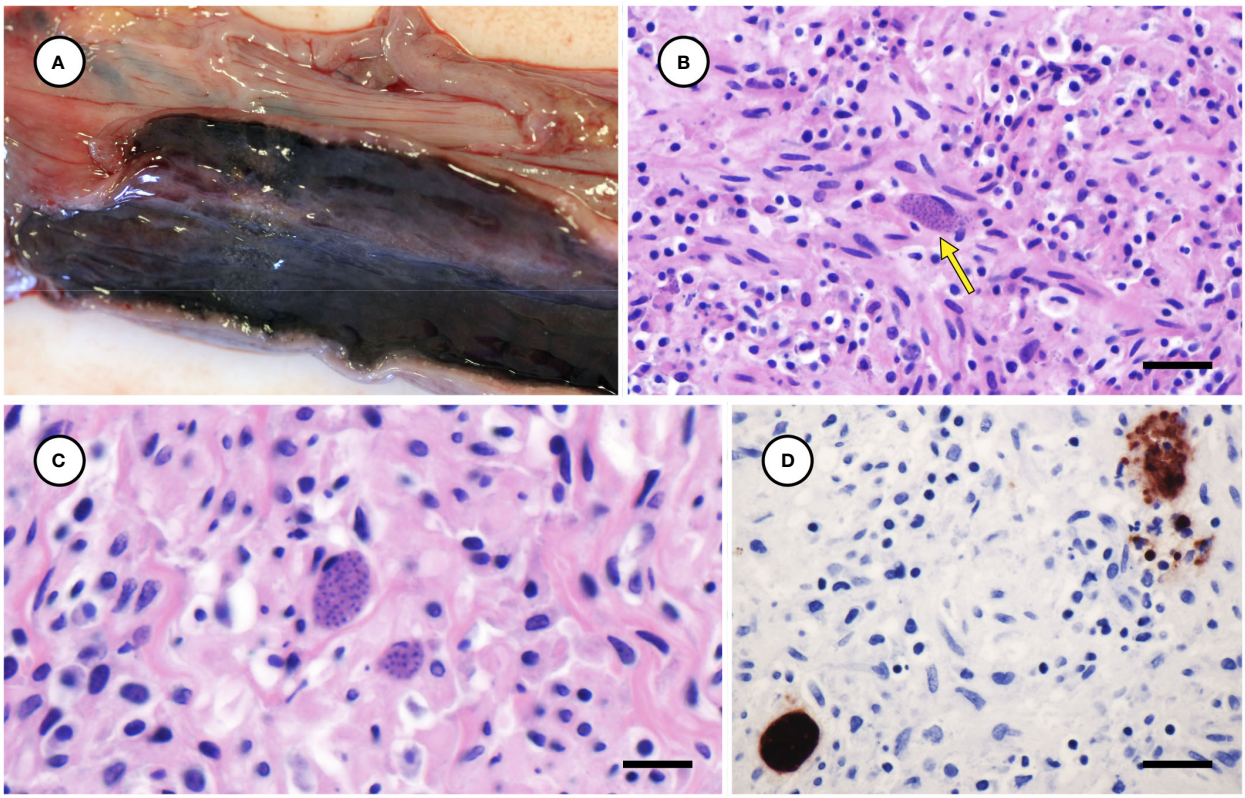

FIGURE 8
Mildly enlarged and congested left uterine horn from a sea otter with fatal toxoplasmosis and steatitis **(A)**. Low **(B)** and high **(C)** magnification microscopic views of severely inflamed uterine myometrium from the same otter with intralesional *Toxoplasma gondii*-like tissue cysts (indicated by arrow for **B**) (**B, C**: H&E stain, bar = 20 and 10 µm, respectively). Immunohistochemical stain of uterine myometrium from the same sea otter demonstrating strong positive immunostaining of intralesional protozoa for *T. gondii* **(D)** (Bar = 20 µm).

Australian marsupials (Canfield et al., 1990), and HIV-positive humans with protozoal pneumonia and gastritis (Nash et al., 1994; Alpert et al., 1996).

Although co-infection with *S. neurona* was identified via parasite isolation, immunohistochemistry, or PCR for two cases (Supplementary Table 1), findings from extensive histopathology and immunohistochemistry confirm *T. gondii* as the primary cause of death in all four cases. All otters for which pericardial fluid was available for serology had high positive titers for *T. gondii* and low positive titers for *S. neurona*. *Sarcocystis neurona* was isolated in culture from the brain of Case 2; however, no *S. neurona* organisms were seen associated with inflammation in the brain histologically or immunohistochemically. While Case 4 exhibited sparse *S. neurona* immunopositive staining in heart, adipose and lung, DNA extracted from the brain, subcutaneous fat, omentum, lung, and spleen for this sea otter only yielded amplification at the ITS1 locus for *T. gondii*. As the parasite load of *T. gondii* was far greater and was closely associated with pathology in multiple tissues from all examined otters, *S. neurona* appeared to be incidental or of minimal clinical significance. However, it cannot be ruled out that *S. neurona* co-infection may have contributed to the severity of observed lesions.

This study had several limitations including a small sample size, varying postmortem duration, and freeze-thaw conditions for examined animals, which can decrease tissue quality and resolution. Additionally, steatitis altered the gross appearance of adipose stores and likely impacted standardized assessment of nutritional condition.

Our findings reveal a novel and concerning lesion pattern for southern sea otters with systemic toxoplasmosis that appears to be associated with an atypical *T. gondii* strain described in an aquatic animal for the first time. The unique combination of host, pathogen, and environmental factors contributing to this newly recognized manifestation of systemic toxoplasmosis in sea otters is unknown. Future directions should include expanded research on this seemingly highly pathogenic *T. gondii* strain, such as genomic studies to identify unique virulence factors, or transcriptomic investigations into host lipid metabolism and tropism. Additional studies are needed to evaluate the presence of this strain in a larger sample of coastal terrestrial animals, sea otters, and other aquatic wildlife, including screening animals with subclinical *T. gondii* infections. Investigating habitat or climate change factors that may have facilitated the spread of this rare atypical strain to coastal California will shed light on how anthropogenic activities can alter *T. gondii* ecology and epidemiology. The potential for immunosuppression following exposure to occult viruses, toxins, anthropogenic chemicals, and other causes also warrants investigation. Because *T. gondii* oocysts can be concentrated in invertebrates and transmitted to susceptible hosts via ingestion (Arkush et al., 2003), there is potential for this virulent pathogen to pose a public health risk for people consuming seafood, or ingesting water contaminated with this parasite. Sea otters have served as important sentinels for recognition of the COUG strain circulating in the coastal California terrestrial and marine

TABLE 3 Findings from molecular characterization using PCR and MLST for southern sea otters (*Enhydra lutris nereis*) with fatal *Toxoplasma gondii*-associated steatitis and toxoplasmosis.

Case number	Tissues tested	ITS1 amplification (identity)	cox1 amplification (identity)	B1 amplification	MLST[a] amplification	*Toxoplasma* genotype[b]
Case 1	Subcutaneous adipose	.	.	+	+	COUG
	Spleen	.	.	+	+	.
	Lung	.	.	+	.	.
Case 2	Lung	.	.	+	.	.
	Subcutaneous adipose	.	.	+	+	COUG
	Spleen	.	.	+	+	.
	Omentum	.	.	+	.	.
	Brain isolate (no trypsin)	+ (*T. gondii*)	+ (*S. neurona*)	.	.	.
	Brain isolate (with trypsin)	+ (*T. gondii*)	–	+	+	COUG
Case 3	Mesentery	.	.	+	.	.
	Omentum	.	.	+	.	.
	Brain	.	.	+	+	.
	Subcutaneous adipose	.	.	+	+	COUG
	Heart	.	.	+	.	.
Case 4	Brain	+	.	+	+	.
	Subcutaneous adipose	+ (*T. gondii*)	.	+	+	COUG
	Omentum	+	.	+	.	.
	Lung	+	.	+	.	.
	Spleen	+	.	+	.	.

"." = Not done.
[a]= MLST includes 12 loci: SAG1, 5'SAG2, 3'SAG2, altSAG2, SAG3, BTUB, GRA6, C22-8, C29-2, L358, PKI, and Apico.
[b]= See Supplementary Figure 2 for detailed sequencing results.

environment, highlighting the need for ongoing research into spread and possible emergence of diverse *T. gondii* strains from coastal terrestrial vertebrates to marine ecosystems.

Data availability statement

The data presented in the study are deposited in GenBank, accession numbers: OQ249665 (3'SAG2), OQ249666 (5'SAG2), OQ249667 (altSAG2), OQ249668 (Apico), OQ249669 (B1), OQ249670 (BTUB), OQ249671 (C22-8), OQ249672 (C29-2), OQ249673 (GRA6), OQ249674 (L358), OQ249675 (PKI), OQ249676 (SAG1), OQ249677 (SAG3).

Ethics statement

In accordance with Section 109(h) of U.S. Marine Mammal Protection Act (MMPA), U.S. Fish and Wildlife Service (Service) regulations implementing the MMPA at 50 CFR 18.22(a), and in accordance with Service regulations implementing the US Endangered Species Act at 50 CFR 17.21(c)(3), samples used to complete this work were collected by an official or employees of the California Department of Fish and Wildlife (CDFW) in the course of their duties as an official or employee of CDFW.

Author contributions

MM: Completed gross necropsies for some enrolled otters; read out all case slides and compiled summary reports; envisioned, coordinated, and oversaw project completion; took gross photos and all photomicrographs; led completion of all summary image plates; substantial contribution to manuscript design, preparation, revision, editing and literature review; designed and contributed Tables 1, 2; Supplementary Figure 2, and Figures 1 through 8. CN: Leadership role in manuscript writing, editing, and polishing of case discussion and literature review; helped coordinate and oversee project completion; assisted with gross necropsy for one enrolled

otter and transported samples to UCD for diagnostic workup; provided substantial input regarding project design and completion, and manuscript design, preparation, revision, editing and literature review; final formatting and polishing of manuscript to meet publication guidelines. DS: Extracted DNA and performed PCR for all *T. gondii* genotyping assays for enrolled otters; established and maintained *in vitro* cultures for one otter and verified culture ID via PCR; assisted with sequence trimming and alignment; assisted with design and construction of Table 2; reviewed all histopathology slides and immunohistochemistry; designed and contributed Table 3 and Supplementary Figure 2; assistance with literature review, and manuscript writing and editing, especially sections of the discussion related to genotyping and the COUG *T. gondii* strain. FB: Assisted with gross necropsies, data, and sample collections; coordinated specimen subsampling and shipments, and immunohistochemistry shipments for diagnostic workup; conducted database queries of existing southern sea otter steatitis data; data organization; assisted with manuscript edits and submission. KG: Assisted with gross necropsies, data, and sample collection; coordinated archiving of samples and data to assist completion of summary reports; completed histopathology trimming of 2/4 enrolled otters; assisted with manuscript edits and table completion. AR: Assisted with histopathology trimming of 2/4 enrolled otters; assisted with manuscript edits and completed reformatting and optimization of all image montages for the manuscript. CY: Provided critical field recognition of cases enrolled in the study; assisted with gross necropsies, data, and sample collection; assisted with manuscript edits. Completed gross necropsy on 1 case. MH: Provided critical field recognition of cases enrolled in the study; assisted with gross necropsy for 1 case, data, and sample collection; assisted with manuscript edits. Created Supplementary Figure 1. AP: Completed all serological testing for *T. gondii* and *S. neurona* for three cases and provided expertise to facilitate parasite isolation efforts; assisted with manuscript edits. KS: Supervised and funded parasite tissue isolation and protozoal parasite molecular detection and characterization at UC Davis; analyzed sequence data and interpreted molecular results; substantial assistance with literature review and suggestion of applicable manuscripts on genotyping details; substantial assistance with manuscript writing and editing, especially sections of the discussion related to genotyping and the COUG *T. gondii* strain. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1116899/full#supplementary-material

SUPPLEMENTARY TABLE 1
Findings from histopathology, immunohistochemistry, and serology for southern sea otters (*Enhydra lutris nereis*) with fatal *Toxoplasma gondii*-associated steatitis and toxoplasmosis.

SUPPLEMENTARY FIGURE 1
Map of the central California coast, showing the stranding locations of four southern sea otters (*Enhydra lutris nereis*) with fatal *Toxoplasma gondii*-associated steatitis and toxoplasmosis.

SUPPLEMENTARY FIGURE 2
Heat maps comparing the sequence identity of *Toxoplasma gondii* DNA amplified from the four sea otters with systemic toxoplasmosis and reference genotypes including the COUG strain, Type X (bobcat) and the archetypal Types I (RH), II (ME49), and III (CTG). Darker shades of orange indicate higher percent identities between the *T. gondii* strain infecting sea otters and reference strains across the 13 examined loci.

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Strengthening the health surveillance of marine mammals in the waters of metropolitan France by monitoring strandings

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Monitoring the health status of marine mammals is a priority theme that France aims to develop with the other European Union Member States in the next two years, in the context of the Marine Strategy Framework Directive. With approximately 5,000 km of coastline and for nearly ten years, France has been recording an average of 2,000 strandings per year, which are monitored by the National Stranding Network, managed by Pelagis, the observatory for the conservation of marine mammals from La Rochelle University and the French National Center for Scientific Research. Since 1972, this network has successively evolved from spatial and temporal faunistic description to, nowadays, the detection of major causes of mortality. It now aims to carry out epidemiological studies on a population scale. Thus, a strategy to strengthen the monitoring of marine mammals' health status based on stranding data has been developed. This strategy will allow for a more accurate detection of anthropogenic cause of death as well as those of natural origin. It will allow the monitoring of time trends and geographical differences of diseases associated with conservation and public health issues while ensuring the early detection of emerging and/or zoonotic diseases of importance. It will also allow a better assessment of the consequences of human activities on these animal populations and on the environment. Thus, this strategy is fully in line with the "One Health" approach which implies an integrated vision of public, animal and environmental health. It is broken down into four surveillance modalities: (1) general event-based surveillance (GES); (2) programmed surveillance (PS); (3) specific event-based surveillance (SES); (4) and in the longer term, syndromic surveillance (SyS). This article describes the French strategy as well as these different surveillance modalities, the levels of examinations and the associated sampling protocols and finally, the method of

standardisation of the data collected. The objective is to present the strategy developed at the French level in order to integrate it into a future strategy shared at the European level to standardise practices and especially complementary analysis, necessary for a better evaluation of the health status of these mobile marine species.

KEYWORDS

marine mammals, health surveillance, eco-epidemiology, One Health-strandings, epidemiosurveillance network, conservation, wildlife, metropolitan France

1 Introduction

Epidemiological surveillance of wildlife consists of monitoring the health status and risk factors in these animal populations (Toma et al., 2010). Improved knowledge allows measures to be taken to face major issues such as the preservation of biodiversity, the conservation of vulnerable species, the maintenance of the economy associated with domestic animals and the protection of public health. It is thus recognised that wildlife epidemiosurveillance needs to be better integrated into the One Health approach (Karesh and Cook, 2005; Buttke and Wild, 2014; Cunningham et al., 2017; Sleeman et al., 2017), which involves an integrated view of public, animal and environmental health. Many species of terrestrial wildlife are indeed responsible for zoonosis. For example, Eurasian badgers (*Meles meles*) in the United Kingdom and Ireland and wild boars (*Sus scrofa*) in Spain, are reservoirs of *Mycobacterium bovis*, which are involved in the transmission of Bovine tuberculosis to cattle (Réveillaud et al., 2018). Another example of pathogen's transmission between wildlife and domestical animals is the canine distemper virus in the lion (*Panthera leo*) population of the Serengeti National Park in Tanzania. This virus is similar to the one circulating in domestic dogs (*Canis lupus familiaris*) and the contacts between these two species make it possible to transmit the virus between them (Cleaveland et al., 2000; Viana et al., 2015). In addition, terrestrial wildlife is very present in the cities and could also be responsible for the transmission of zoonosis. Rodents such as the Norway rat (*Rattus norvegicus*) are known to be intermediate hosts of alveolar echinococcosis and since 1996, the red fox (*Vulpes vulpes*) has been described as one of the definitive hosts of this parasite. These species present in our cities are therefore likely to transmit this parasitosis to humans through their faeces (Bresson-Hadni et al., 2004; Vuitton et al., 2010).

Unlike terrestrial wildlife, the interactions between the health of marine mammals and human or domestic animal health may seem less obvious, given the more limited contact with marine mammals (Crespo and Hall, 2001). Moreover, difficulties are met to conduct the monitoring on marine wildlife, as access to these animals is difficult because of their habitat and their behavior (i.e., cryptic species, highly mobile species, diving behavior). Nevertheless, marine mammals should be considered as sentinel species not only for the health of the oceans but also of humans' health (Bossart, 2011). Thus, the monitoring of strandings represents a valuable source of data to inform conservation management (IJsseldijk et al., 2020b). In addition to the spatiotemporal mortality trends and the cause of death, necropsies and complementary analysis carried out on stranded animals allows to understand the circulation of pathogens (bacteria, viruses, parasites and fungi) in these animal populations (e.g. Stokholm et al., 2021) which can constitute a conservation or public health issue (Ossiboff et al., 2021). In addition to detecting pathogens that would cause the death of the animal, it is possible to identify pathogens carried by an animal without being responsible for the death, or to detect antibodies, which would provide evidence that the targeted agent is circulating in the population (Bodewes et al., 2015; Measures and Fouchier, 2021). Laboratory analysis can also detect phenomena such as antibiotic resistance in isolated bacteria which is of public health interest (e.g. Gross et al., 2022). As with terrestrial wildlife, determining the causes of mortality and monitoring the health status of marine mammals ultimately makes it possible to identify the main threats to these animals and to respond to a public health issue, particularly with the early detection of zoonotic agents (Kuiken et al., 2005).

Therefore, many coastal countries in Europe and around the world have set up systems to record strandings and carry out *post-mortem* examinations of carcasses, thus taking advantage of these strandings to improve scientific knowledge (Perrin and Geraci, 2009). In Europe, some of these networks are effective for health surveillance and carry out necropsies with additional analysis for diagnostic purposes, such as in Germany (Institute of Wildlife and Marine Research of the University of Hanover), in Netherlands (Faculty of Veterinary Medecine, Department of Biomolecular Health Sciences, Division of Pathology, Utrecht University), in Great Britain (UK Cetacean Stranding Investigation Programme and ZSL Institute of Zoology), in Belgium (Faculty of Veterinary Medicine in Liège), in Italy (National Reference Center for Diagnostic Activities on Stranded Marine Mammals) and in Spain (University of Valencia, University of Las Palmas de Gran Canaria). These efforts have led to numerous publications that make it possible to assess the health status of populations at least on a regional scale (e.g. Prenger-Berninghoff et al., 2008; Arbelo et al., 2013; Mahfouz et al., 2014; van de Velde et al., 2016; Kershaw et al., 2017; Pintore et al., 2018; Coombs et al., 2019; Kapetanou et al., 2020; Numberger et al., 2021; Audino et al., 2022). Routine analysis are also carried out, but apart from a few main pathogens known in marine mammals, they often

differ from one country to another (Guillerit, 2017). This is why standardisation of analysis between countries would allow the results obtained to be inferred at the population level of these widely distributed mobile species. This is all the more important as there are other considerations to be taken into account when conducting health monitoring of marine mammals. They are protected species covered by numerous regional and international directives and conventions. At the international level, these are mainly the Convention on the Conservation of Migratory Species of Wild Animals (CMS, Bonn Convention) and the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). In the North East Atlantic, the OSPAR (Oslo-Paris) convention and ASCOBANS (Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Seas) both cover the conservation of marine mammals. These species are also regulated in French law through the decree of the 1st of July 2011 in application of the CITES Convention (Washington Convention) and Directive 92/43/EEC (European Directive known as the Habitats-Fauna-Flora Directive). Furthermore, at European level, the 2008/56/EC Marine Strategy Framework Directive (MSFD) published on the 25th of June 2008 and transposed in France in a series of regulations concerning the marine environment, requires knowledge of the health status of marine mammal populations to be improved.

The French National Stranding Network (NSN) is coordinated by the Pelagis observatory in La Rochelle (Support and Research Unit, UAR 3462 La Rochelle University - French National Center for Scientific Research), appointed by the Ministry of Ecology to monitor the status of marine mammal populations and support the implementation of public conservation policies relating to these species. The monitoring of strandings makes it possible to meet the requirements linked to the regulated status of these species. For example, among the descriptors defined by MSFD and used to define Good Ecological Status (GES), marine mammals are involved in descriptor D1 "Biodiversity" and descriptor D8 "Contaminants" with the following expectations: (1) monitoring of coastal cetacean populations, (2) monitoring of seal populations, (3) monitoring of marine mammals at sea, (4) monitoring of marine mammal strandings, (5) monitoring of interactions with human activities, (6) monitoring of chemical contaminants in cetaceans. Thus, the monitoring of strandings is an integral part of this monitoring.

The NSN was created in 1972 as a natural science network. Since its beginnings, the network has relied on the participation of volunteers (correspondents), but over time it has become partially professional, with some correspondents such as field agents of the French Biodiversity Agency (*Office Français de la Biodiversité - OFB*) for whom this activity is part of their missions. The network has evolved from a descriptive activity of stranded animals to the detection of the main causes of mortality through the examination and necropsy of some selected carcasses and the study of the ecology of species through various samples and data analysis. From this point of view, this network is particularly functional and generates approximatively twenty publications per year in the field of ecology (e.g. Spitz et al., 2014; Peltier et al., 2021; Chouvelon et al., 2022; Méndez-Fernandez et al., 2022; Rouby et al., 2022). Certain zoonotic pathogens (*Brucella* sp. and *Erysipelothrix rhusiopathiae*) and epizootic agents (morbillivirus and avian influenza virus) are only investigated if macroscopic lesions are suggestive of infection by these pathogens. In this global context, Pelagis has developed a strategy to better monitor the health of marine mammals based on stranded marine mammals. To this end, it is necessary to develop the network and strengthen the system for monitoring the health of marine mammals in order (1) to harmonise the methods used to diagnose and identify the causes of mortality with greater accuracy (2) to improve the understanding of the main threats to marine mammals, particularly emblematic or critical species (because of their rarity or the associated conservation issue) as well as strandings associated with phenomena of interest (e.g. unusual mortality events); (3) to improve the ability to analyse data through epidemiological models (4) to allow the acquisition of knowledge on the circulation of pathogens in these populations and their impact on animal and public health in case of zoonosis; (5) to allow the early and easy detection of peak mortality and other monitored factors; and finally, (6) to allow the early detection of major (re-)emerging, exotic or zoonotic diseases. Thus, this strategy falls within the field of eco-epidemiology while integrating the "One Health" approach.

The French stranding network provides a case study: the conceptual framework will be confronted with reality, considering the objectives, structure, functioning and tools of the NSN, which will need to evolve further to better meet the requirements of the strategy. The limits and needs will be identified as well as the long-term prospects. The objective is to present the strategy developed at the French level (metropolitan and overseas) in order to integrate it into a future strategy shared at the European level to standardise practices and especially complementary analysis, necessary for a better evaluation of the health status of these mobile marine species.

2 Preliminary considerations for strategy development

2.1 Functioning of the NSN

The NSN is structured in a similar way to an epidemiological surveillance network, with the different levels of organisation being field, local, regional and central (Dufour and Hendrikx, 2011) (Figure 1).

The national governance of the NSN is ensured by a Steering Committee (COPIL) made up of appointed members from different institutions (Pelagis, OFB, surveillance network for infectious diseases of birds and wild terrestrial mammals (SAGIR), National Museum of Natural History (NMNH), ministry in charge of Ecology), divers non-profit associations, members elected from among the NSN correspondents and invited experts. With the exception of the French Overseas Territories, coordination is carried out solely at national and not regional level, but depending on the region, data are sometimes centralised by a local representative correspondent. Technical and scientific

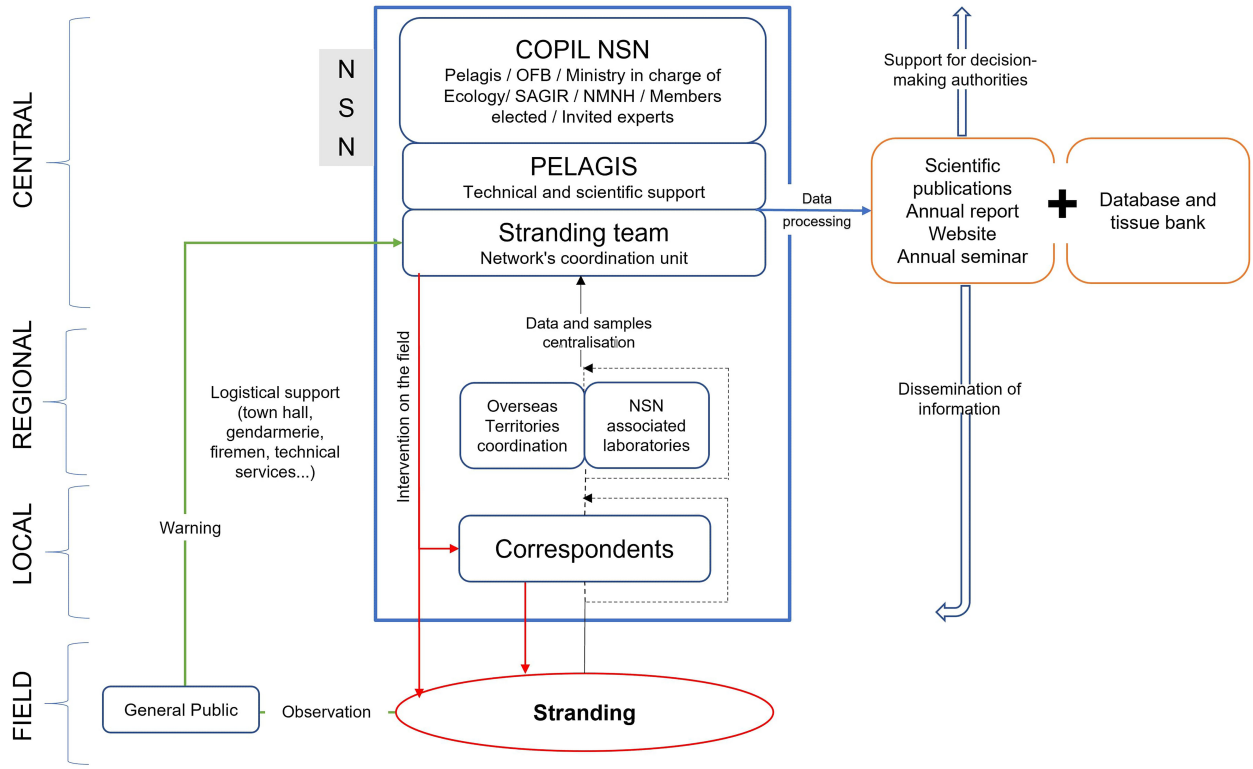

FIGURE 1
Organization of the NSN.

support is provided by Pelagis. The network's coordination unit, known as the "stranding team", is located within Pelagis. To date, only two laboratories have signed agreements at regional level (LABOCEA in Ploufragan and Department of Morphology and Pathology of the Faculty of Veterinary Medicine in Liège).

Carcass data are collected in the field according to standard protocols by authorised NSN correspondents or in NSN associated laboratories. These authorisations (called "green card") are allocated by the ministry in charge of Ecology, subsequent to the training delivered by Pelagis. The application of the various protocols (biometrics, standardised photographs, external examination, internal examination, necropsy and sampling) will depend on the state of decomposition of the animal, the level of training of the correspondent and the logistical means available (transport and storage of the carcass, storage of samples, etc.) (Table 1). The DCC, "Decomposition Code Categories", is the indicator of the state of decomposition of the carcass used, which allows a score to be assigned from 1 to 5, from the freshest to the most putrefied respectively (Kuiken and García Hartmann, 1991; Jauniaux et al., 2002; Van Canneyt et al., 2015).

All the information is finally centralised by the stranding team and integrated into a database. The data, once standardised, are made available for studies by external entities. A tissue bank is also set up and available for research projects. Each year, approximatively thirty agreements for the use of biological samples are drawn up with external organisations. The data analysis and the studies' results carried out by the observatory itself give rise to scientific publications, the annual stranding report, publications on the website and are also presented at an annual seminar which brings together the network's correspondents and stakeholders.

Finally, the annual operating budget of the network managed by Pelagis can be divided into four main components: scientific and administrative coordination, human resources, interventions and sample analysis. The ministry in charge of ecological transition, as well as the supervisory institutions of Pelagis, which are the French National Center for Scientific Research and La Rochelle University, funds the coordination of the NSN. The stranding team and the interventions are mainly supported by the OFB and La Rochelle city. Depending on the year, the budget allocated to sample analysis varies and is supported by the OFB, the ministry and regional, national or European projects. The budget managed by Pelagis for stranding activities is estimated between €400,000 and €600,000 per year, depending on the projects integrating sample and data analysis. Finally, a large part of the NSN's operation is ensured by the self-financing of the network's stakeholders, which can be broken down as follows: valuation of the correspondents' voluntary work, participation of non-profit organizations making use of their financial resources, provision of OFB agents, technical services of municipalities, prefectures and other structures, provision of premises or equipment by these same structures.

TABLE 1 Summary of the examination levels and sampling protocols.

			Data on individual's health and cause of death	Biological, ecological, demographic and contaminant data	Maximal DCC
Examination type					
Levels	L1	External examination	Body condition Description of macroscopical external lesions	Species Sex Biometrics	3
	L2	External examination + partial internal examination	Body condition Description of macroscopical external lesions Basic data on some main organs (lungs, stomach, spleen, liver)		
	L3	External examination + complete internal examination	Body condition Description of macroscopical external and internal lesions		
	L4	Necropsy (by a trained veterinarian)	Body condition Diagnostic and interpretation of macroscopical external and internal lesions (necropsy performed by an experienced veterinarian, maximal level of examination)		
Sampling					
Protocols	P1	Teeth	–	Age	5
	P2	P1 + blubber, muscle, stomach, liver, kidney, gonads	–	Age Reproductive status Diet Level of contamination	3
	P3	P2 + lymph nodes, blubber, muscle, spleen, pancreas, stomach, intestine, liver, adrenal gland, kidney, bladder, thyroid, thymus, heart, lungs, brain, tympanic bulla, foetal tissues, parasites	Systematic and standardised analyses: microscopical lesion (histopathological analysis) and pathogens (bacteriological agents, virus, mycotic agents, toxins, parasites)	Age Reproductive status Diet Level of contamination	2
	P0	Different for each case	Analyses for diagnostic purposes, non-standardised, based on a specific context and/or macroscopical lesions	–	3

2.2 NSN's stranding valuation tools: examinations and samples

There are different tools for valuing strandings that correspond to the levels of examination and sampling protocols carried out on the carcasses. Thus, depending on the operator and his training, four levels of examination and three standardised sampling protocols can be carried out (Table 1). Although it would be desirable to carry out the highest level of examination and sampling protocol on each carcass found, the very large number of annual strandings on the French coasts does not allow this at present, for logistical and financial reasons. The levels of examination and associations with sampling protocols have been revised and improved during 2022 to better meet the objectives pursued in the framework of the reinforcement of health surveillance.

The first examination level (L1) is an external examination associated to six standardised photographs (Figure 2). It is carried out on the majority of stranded animals and may provide initial information on the cause of death. From now on, the training of the correspondents implies that they are also able to describe precisely the external lesions observed with standard description criteria (location, shape, contours, distribution, size and extension, number, consistency and texture) while completing their examination with photos of the lesions described. Sections are systematically taken at the level of the observed alterations in order to examine and describe the underlying tissues (Figure 3). This makes it possible to determine whether these alterations occurred during the animal's lifetime or after its death and facilitates interpretation.

The second level of examination (L2) used to consist of performing the L1 and opening cavities in a second stage, in order to perform the samples of the P2 protocol, intended mainly for ecological analysis. This level has been significantly modified and from now on correspondents will be trained to describe some main organs (descriptive criteria: volume, shape, colour, consistency, *etc.*). Thus, they must briefly describe the appearance

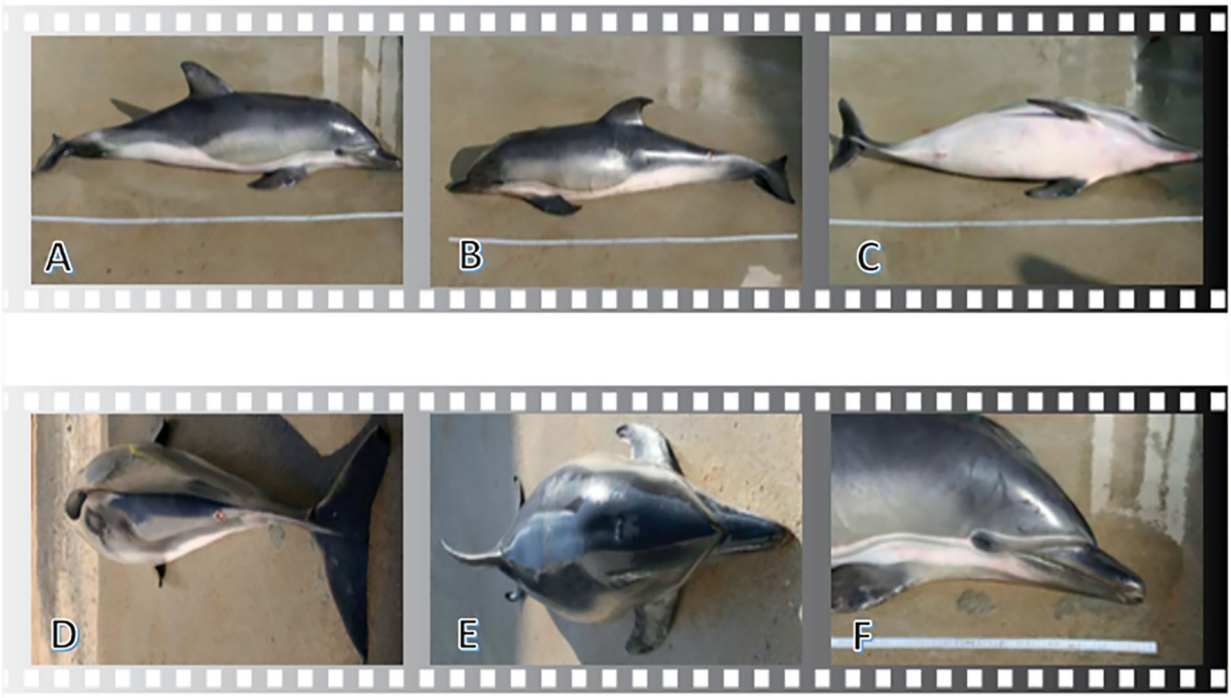

FIGURE 2
The six standardised photos, © PELAGIS. **(A)** Lateral right view; **(B)** Dorsal view; **(C)** Ventral view; **(D)** Rear view; **(E)** Front view; **(F)** Head focus.

of the lungs, the contents of the stomach, the liver, the spleen and finally, they must check the topography of all the thoracic and abdominal organs. These different elements are all photographed with a measurement and colour scale (Figure 4). These organs were chosen because they allow for a more accurate diagnosis of mortality linked to bycatch in fishing gear. Indeed, animals that die from bycatch usually show good body condition, large amounts of fresh or partially digested food remains in the stomach, congestive liver and spleen, lung edema, congestion and/or emphysema and finally, normal topography of the internal organs

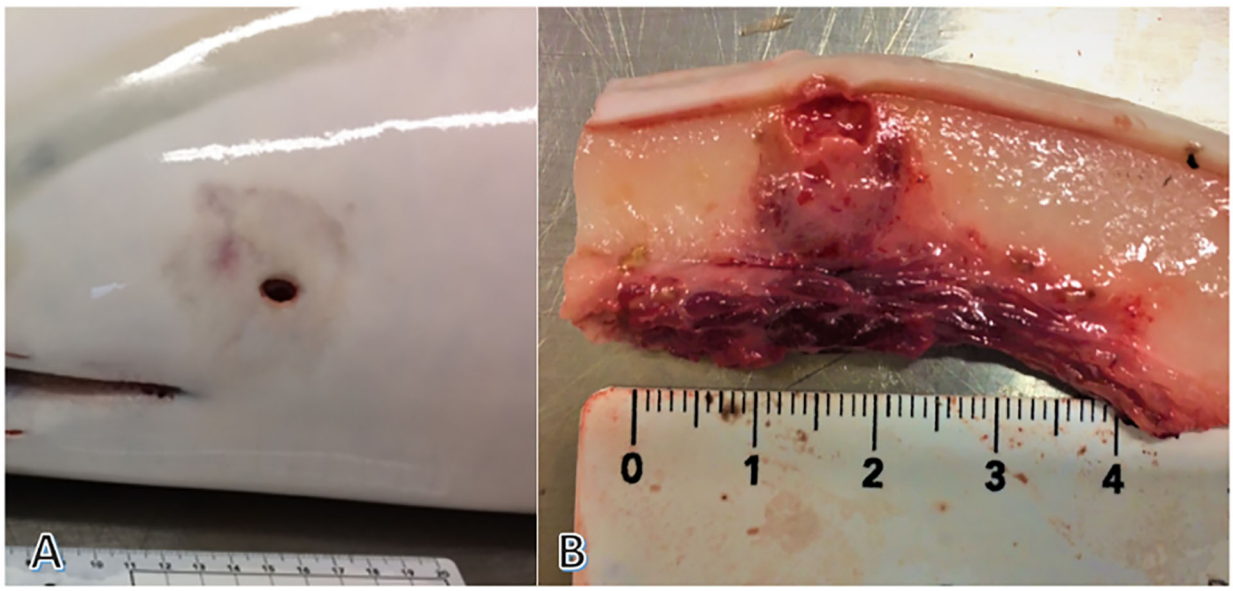

FIGURE 3
Skin lesion in external view **(A)** and cross-section **(B)**, © PELAGIS.

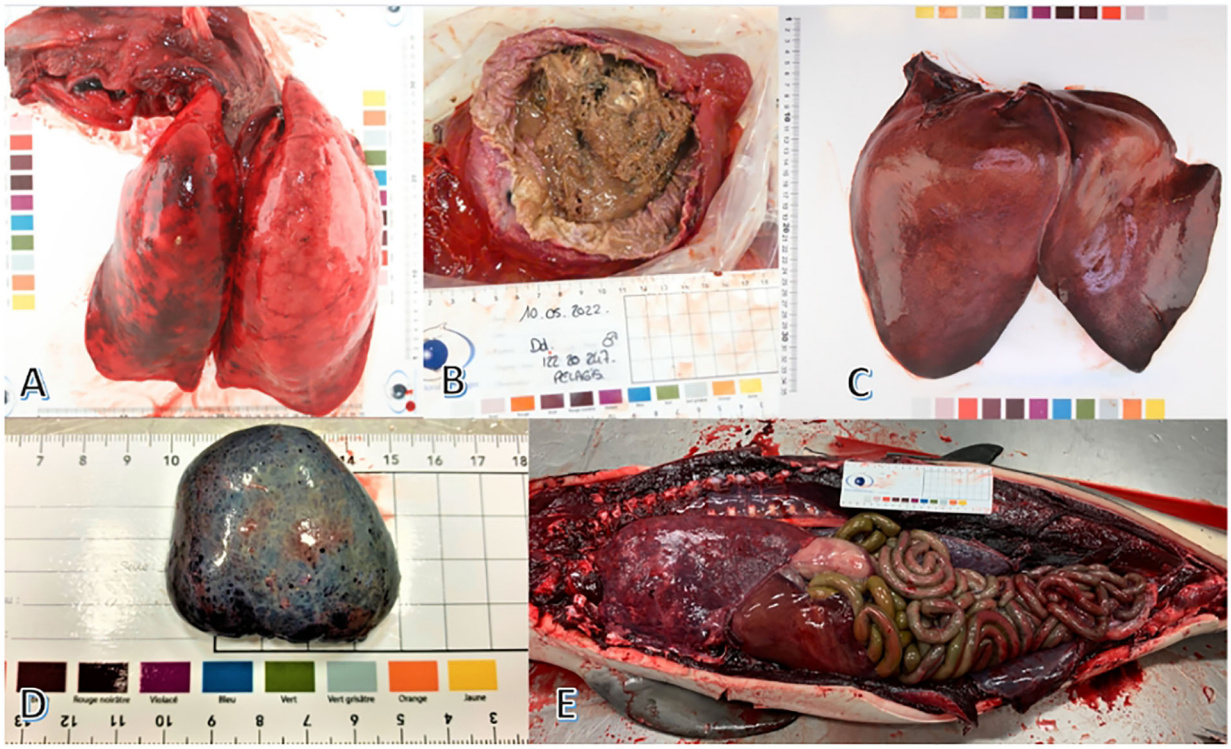

FIGURE 4
Organs described and photographed at L2 in a cetacean, **(A)** lungs, **(B)** gastric contents, **(C)** liver, **(D)** spleen, **(E)** thoracic and abdomen topography, © PELAGIS.

(Kuiken, 1996; de Quirós et al., 2018; Epple et al., 2020; IJsseldijk et al., 2021). This also helps to rule out other major pathology that would be macroscopically visible on the targeted organs.

The third examination level (L3) is divided into two phases. The first step consists in the production of a dissection report by a trained NSN's correspondent (biologist or veterinarian), which includes the external examination and a detailed description of all internal organs, supported by photographs. The second phase is the interpretation and validation of this report by an experienced veterinarian who can then conclude, when possible, on the cause of death. This level has been developed by Pelagis in 2019 as part of a veterinary thesis (Laporte et al., 2021) in a context of judicialization of the problem of bycatch in fishing gear. As the systematic intervention of an experienced veterinarian on all the carcasses of bycaught animals is difficult to consider, it was a priority to find alternative solutions to respond more accurately for the diagnosis of mortality linked to bycatch. This level involves to train operators in the detailed macroscopic description of all the organs and in taking photographs in a standardised way in order to produce an interpretable report. The operators trained to the L3 are non-veterinarian marine biologists, specialized in the monitoring of stranded marine mammals, and volunteered veterinarians with less experience. The inclusion of veterinarians in the L3 allows for systematic cross-checking of their interpretations and conclusions by more experienced veterinarians, which contributes to the harmonization of diagnosis.

The fourth examination level (L4) corresponds to a necropsy performed by an experienced veterinarian with consolidated knowledge of marine mammal pathology and who has performed numerous necropsies on these species. The necropsy is performed according to an adapted standard protocol (IJsseldijk et al., 2020a). L4 is the level with the best diagnostic value and generally allows the determination of the cause of death.

In addition to the *post-mortem* examinations carried out, there are different sampling protocols for various analysis that can be carried out depending on the level of examination conducted (Table 1). The P1 sampling protocol consists of taking the teeth to determine the age of the animal. This sampling protocol may be carried out on all animals regardless the DCC. The P2 sampling protocol consists of taking different samples in addition to P1 in order to acquire biological, ecological, demographic and contaminant data. It can be carried out on animals of up to DCC 3. A new standard sampling protocol recently defined, the P3, which is added to P1 and P2 protocols, is intended for epidemiological surveillance and can only be carried out on animals of DCC 1 or 2. It was defined according to several criteria: improvement of knowledge, conservation and public health interests, financial cost, technical and logistical feasibility. The number of organs taken for standard formalin samples for histopathological analysis is 23 (or more if the animal is pregnant). Of these, 11 only are analysed as part of research projects in order to limit the financial cost. For example, the thyroid and adrenal glands are systematically

sampled but not analysed, but could be analysed at a later time in the context of projects looking at the impact of contaminants on the endocrine system. The organs whose samples are systematically analysed are some main vital organs (heart, brain, liver, spleen, kidneys, pancreas, intestine and lungs) and the lymph nodes (with the exception of the marginal lymph node in cetaceans, which is taken in addition to the tracheobronchial nodes for the respiratory system and which is only analysed on a project basis or at the specific request of the veterinarian). Moreover, six standard refrigerated samples are taken for bacteriological analysis (spleen, liver, heart, lungs, kidneys and brain). The samples are inoculated on various culture media in different atmospheres to test for aerobic and anaerobic bacteria. Specific culture media may be used when the veterinarian suspects the presence of bacteria requiring it, such as *Salmonella* spp. or *Erysipelothrix rhusiopathiae*. Antibiotic susceptibility testing are performed on bacteria on the list of antibiotic-resistant "priority pathogens" (Tacconelli and Magrini, 2017; Tacconelli et al., 2018). Standard samples are frozen for routine molecular analysis. Ten organs are systematically sampled (lymph nodes, spleen, intestinal segment, liver, kidney, lung and brain) to test for morbilliviruses, influenza viruses, herpesviruses, *Brucella* sp. and finally mycotic agents by (RT-)PCR. If the signal is positive, sequencing is performed to confirm the signal and determine the pathogen involved. Faeces and urine samples are also frozen for future test for algal biotoxins. Samples of parasites found are systematically preserved in ethanol for identification. Finally, upon receipt of the histology results, additional tests for pathogens other than those routinely sought may be requested by the veterinarian (PCR, immunohistochemistry, etc.).

In addition to the standardised protocols, P1, P2 and P3, other samples for diagnostic purposes (P0) may be taken at the request of Pelagis or by the veterinarian in charge of the necropsy, who will then determine the analysis to be performed.

2.3 NSN work focus: strandings and causes of mortality

Reported stranding numbers are the result of four parameters: species abundance at sea, mortality, drift conditions and reporting. The first two are biological variables, while the next two depend on weather conditions and carcass buoyancy, and the last one on the probability of sighting and the observer's ability or willingness to report the stranding (Van Canneyt et al., 2015). To date, the reporting rate in metropolitan France is very high (events are regularly reported several times by different people) and it is considered that almost all strandings are detected (Authier et al., 2014).

The first factors recorded are the specie and sex identification, the biometric data and spatial and temporal distributions of the stranded marine mammals, allowing to follow the trends of the stranding rates and to highlight unusual mortality events. Indeed, changes in these parameters can be indicative of modifications in abundance, mortality, distribution or of pressures affecting marine mammal populations. Diversity within the marine mammal community can also be assessed (Chan et al., 2017; Dars et al., 2021). The external examination of carcasses allows in some cases to suspect the causes of mortality. This is the case, for example, of an animal showing morphological alterations caused by interactions with fishing gear (net imprints, linear lesions encircling the rostrum, mandibular fractures, *etc.*) or even amputations of appendages or perforations carried out during untangling operations (Figures 5, 6).

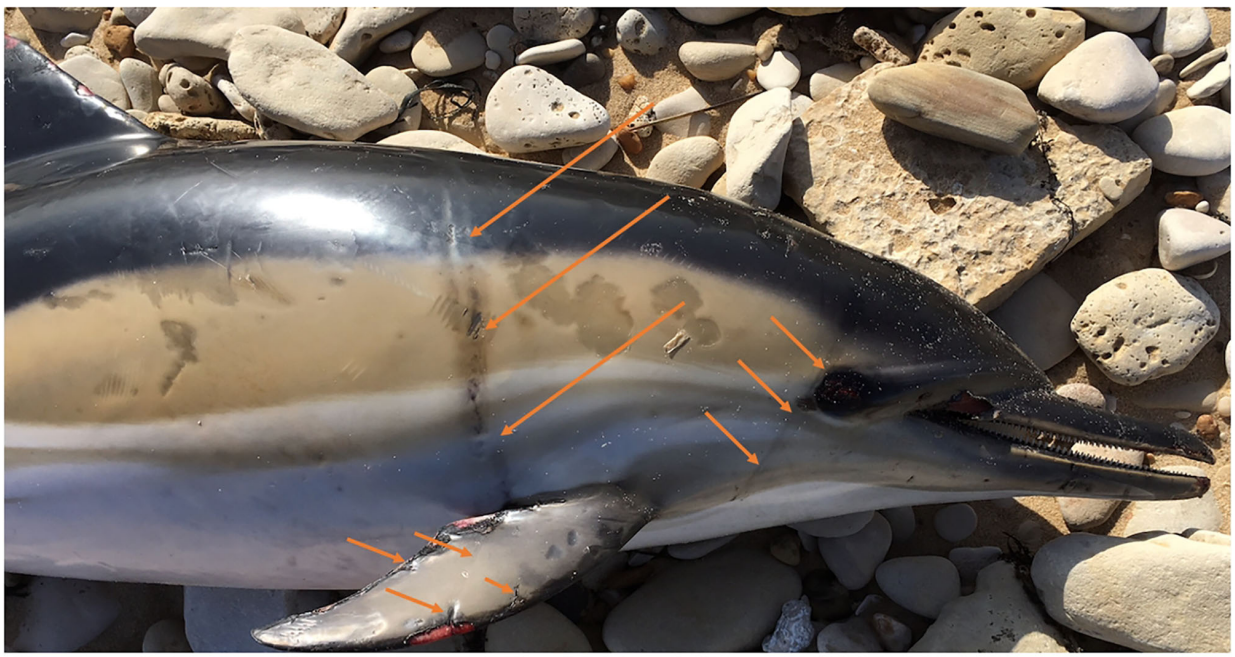

FIGURE 5
Morphological alterations caused by interactions with fishing gear (net imprints) – Common dolphin, February 2019, Rivedoux (17), © PELAGIS.

FIGURE 6
Amputations of appendages and perforations carried out during stripping operations – Common dolphin, March 2022, Bretignolles-Sur-Mer (85), © PELAGIS.

Nevertheless, only a necropsy (*post-mortem* examination by a trained veterinarian) accompanied by additional analysis (e.g. histology, microbiology, virology, parasitology) can lead to a definitive veterinary diagnosis. Necropsy protocols recommand the best practices for cetacean post mortem investigation and tissue sampling for additional analysis (Jauniaux et al., 2019; IJsseldijk et al., 2020a). This allows the cause of death to be determined with higher degrees of certainty than an external examination alone.

According to the stranding database and the annual reports produced by Pelagis, more than 2,000 strandings have taken place each year since 2016. More than 60% of strandings have occurred on the Atlantic coast, around 25% on the Channel and North Sea coast and less than 5% on the Mediterranean coast. More than thirty species of marine mammals have already stranded in metropolitan France. However, the vast majority of strandings (percentage 2016-2021) are represented by only eight species: common dolphin (*Delphinus delphis*, 57%), striped dolphin (*Stenella coeruleoalba*, 6%), bottlenose dolphin (*Tursiops truncatus*, 3%), harbour porpoise (*Phocoena phocoena*, 17%), long-finned pilot whale (*Globicephala melas*, 1%), Risso's dolphin (*Grampus griseus*, <0,5%), grey seal (*Halichoerus grypus*, 9%) and harbour seal (*Phoca vitulina*, 6%). Nevertheless, for the last five years, due to the increase of the numbers of common dolphin strandings, the relative proportion of the long-finned pilot whale and Risso's dolphin represented less than 0.5% of annual strandings each (Van Canneyt et al., 2015). In 2021, 2046 strandings were reported to the NSN. Of these reports, nearly 83% of the carcasses were examined by a NSN correspondent, while less than 5% were necropsied, demonstrating the need to increase the number of necropsies (Dars et al., 2021).

The causes of mortality identified through *post-mortem* examinations of stranded marine mammals are numerous. Among them, are the traumatic causes. One cause widely encountered in small cetaceans is bycatch in fishing gear (Read and Murray, 2000; Jauniaux et al., 2002; Peltier et al., 2021). In 2021, it was the main cause of mortality for common dolphins (87% of the external examinations) and harbour porpoises (52%) in France. Bycatch mortality has also been reported for bottlenose dolphins (16%), striped dolphins (13%) and, to a lesser extent, grey (12%) and harbour seals (8%) (Dars et al., 2021). Collisions with ships are a frequent cause of mortality, especially for large cetaceans. In the Mediterranean Sea, where maritime traffic is intense, more than 50 collisions were reported between 1972 and 2017, representing the main cause of mortality identified in large cetaceans (Peltier et al., 2019). Some deaths are also attributed to entanglement with debris or ingestion of macroplastics (Simmonds, 2012; Moore and Barco, 2013) with the recent example of a fatal entanglement of a young harbour seal in Normandy in 2021 (Dars et al., 2021). It is also possible to observe fatal injuries inflicted by other individuals of the same or other species associated with competitive or predatory behaviour. For example, bottlenose dolphins appear to be particularly aggressive with each other and with other small cetacean species (Ross and Wilson, 1996; Dunn et al., 2002; Nery

and Simão, 2009; Gross et al., 2020). Thereby five cases of striped dolphins stranded in the Mediterranean in 2021 showed lesions corroborating this hypothesis (Dars et al., 2021).

Among the causes of natural death are those of infectious origin. In recent decades, numerous pathogens have been isolated from marine mammals worldwide (Gulland et al., 2018). Among the causes of natural death are also those of non-infectious origin such as starvation (which is very often encountered in young animals separated from their mothers), congenital anomalies (Herr et al., 2020; Morell et al., 2022), neoplasms (Newman and Smith, 2006) or dystocia. In 2021, depending on the type of examination carried out on stranded individuals in France (full or external examination only), natural death resulting from a pathological condition concerned between 18% and 34% of cases in cetaceans and between 6% and 25% in seals (Dars et al., 2021). Pathological condition was considered the cause of death when the animals showed advanced emaciation or major macroscopic lesions on postmortem examination, after eliminating lesions suggestive of traumatic death. External examinations of these animals revealed mainly inflammatory skin lesions scattered over the body and internal examinations revealed that the respiratory system was most often affected (bacterial and/or parasitic pneumonia).

Finally, in recent years, live strandings of cetaceans have represented around 5% of strandings in metropolitan France (Van Canneyt et al., 2015). For live stranded cetaceans, the priority action, whatever the origin, is refloating. Refloating animals in good body condition is often successful. For seals, when their survival depends on it, they are firstly taken care of by a rehabilitation center (mainly first-year individuals, recently weaned or unweaned and separated from the mother).

In 2022, the French news highlighted several cases of out of habitat marine mammals, notably a killer whale (*Orcinus orca*) observed in the Seine River in May followed by a beluga whale (*Delphinapterus leucas*) in August. These cases of out-of-habitats animals often remain unexplained, and there may be multiple reasons for them, such as pathological condition, age (sub-adults disperse more easily), social isolation or environmental conditions (Hennessy et al., 2001; Pryce et al., 2002; Stephens et al., 2005; Clutton-Brock and Huchard, 2013; Vetulani, 2013; Massen et al., 2015; Thompson et al., 2017).

3 Strategy developed and data standardization

3.1 Strategy developed: four health surveillance modalities

In order to meet the objectives of Pelagis and to optimise the use of data obtained from strandings, the strengthening of health monitoring will be based on four methods (Table 2), which may occasionally be supplemented by *ad hoc* surveys. These four methods are: (1) general event-based surveillance (GES), which will apply to all stranded individuals; (2) programmed surveillance (PS), also called active surveillance and defined by the World Health Organization as the collection of case study information as a continuous pre-organized process (World Health Organization, 2002) – the methodology must allow the results found to be inferred to the monitored population and will apply to a sample of 100 individuals representative of the stranded marine mammal population; (3) specific event-based surveillance (SES), which will apply to situations requiring in-depth investigations; and finally, (4) syndromic surveillance (SyS), which will also apply to all stranded individuals.

3.1.1 General event-based surveillance

General event-based surveillance is the monitoring modality that will focus on stranding as an event. It will be based on spontaneous reporting of strandings by the general public. This monitoring has been carried out by the NSN for nearly 40 years and must be maintained as part of the health monitoring strategy developed, as it may help to identify the causes of mortality. The main parameter measured by this modality will be the spatio-temporal distribution of strandings. For example, using reverse drift models, the presumed area of mortality at sea can be defined, which will make it possible to identify possible threats to these animals in this area and thus obtain hypotheses on the cause of death (Peltier et al., 2020). Continuous monitoring of strandings will help to ensure epidemiological vigilance by highlighting unusual mortality events and sometimes identifying the cause, as well as allowing the detection of anomalies on the carcasses. Epidemiological vigilance will be of priority interest as it will ensure the early detection of emerging and/or zoonotic diseases of major importance. In addition, whenever possible, a carcass examination should be carried out by a NSN correspondent, favouring the highest level of examination. Thus, GES is a surveillance modality that will apply to all reported strandings, regardless of the level of examination and sampling protocol carried out.

3.1.2 Programmed surveillance

Programmed surveillance (PS) is a surveillance modality that will involve individuals being subjected to necropsy (L4) and to the P3 sampling protocol which is specific to this modality.

Logistical and financial constraints limit the application of the PS to 100 fresh carcasses per year (DCC 1 or DCC 2 without freezing). Therefore, a sampling plan is proposed to represent the stranded marine mammal population. This plan describes the number of animals expected for each species, by seaboard and by quarter, proportionally reduced for a total of 100 individuals per year based on the last five years of consolidated data, for which all the data are available (from 2016 to 2020 for the 2023 plan). Any stranded animal that falls within this sampling will have to be handled by the PS modality until the quotas are reached. If the expected number of animals for a species, seaboard and quarter is not reached, the animals will be analysed/investigated in the next quarter.

Systematic necropsies accompanied by standardised analysis will allow descriptive epidemiology to be carried out by revealing the circulation of pathogens in these animals and measuring it in time

TABLE 2 Summary of the four monitoring modalities.

Surveillance modalities	Associated levels and protocols	Concerned strandings	Objectives
General event-based surveillance (GES)	Every level Every protocol	All reported strandings	Monitoring of the spatio-temporal distribution of strandings Highlight of unusual mortality events Acquisition of preliminary data on cause of death
Programmed surveillance (PS)	N4 P3, P0	≈ 100 individuals (according to the sampling plan)	Acquisition of knowledge and data for epidemiological studies Obtaining exhaustive lesion tables and inventory of aetiologies and causes of death Inference of the obtained results to the monitored population
Specific event-based surveillance (SES)	Every level P0	Rare species Emblematic species Associated to phenomena of particular interest Individuals suspected of being infected with diseases of priority interest	Strengthening of surveillance on cases defined as priorities
Syndromic surveillance (SyS)	According to the indicators monitored	Continuous collection, according to the indicators monitored	Early detection via algorithms of expected (or unexpected) phenomena and assessment of the impact (or lack of impact) of a phenomena

and space. The prevalence of some agents (morbillivirus, *Brucella* sp., avian influenza, herpesvirus and mycotic agents) in the species of marine mammals most frequently stranded in metropolitan France will be calculated. The same will be done for the prevalence of bacterias identified from the culture media used routinely, as well as for parasites systematically sampled. In addition to infer the results obtained to a reference population, the additional diagnostic analysis included in P3 will make possible to investigate the etiology of the various lesions encountered and thus to better understand their impact on the health of individuals and therefore of populations.

Moreover, obtaining complete lesion tables and numerous laboratory results makes it possible to carry out analytical epidemiology, in particular by conducting case-control studies to measure the effect of exposure to factors on the incidence of diseases or other events. Through this type of study already conducted abroad, it has been possible for example to demonstrate the existence of a positive correlation between exposure to polychlorinated biphenyls (PCBs) and the incidence of infectious diseases in harbour porpoises in Great Britain (Hall et al., 2006). Such studies could easily be carried out given the important work carried out for several years by Pelagis on contaminants and essential trace elements (Cariou et al., 2021; Chouvelon et al., 2022; Méndez-Fernandez et al., 2022). Finally, systematic sampling allows samples to be made available for future research programmes.

3.1.3 Specific event-based surveillance

Specific event-based surveillance is a monitoring modality in which some stranding events will be investigated in depth to meet conservation, public health or knowledge acquisition objectives. These events are called “cases”. They will be revised each year or more regularly depending on the epidemiological context. This surveillance method will be based on spontaneous reporting of cases at the time of reporting by the general public or by the network’s correspondents when they intervene on the stranding field. The SES has thus been established to allow for enhanced monitoring of the following three main categories of cases, each with specific objectives: (1) enhanced monitoring of the health status of critical species and emblematic species for knowledge or conservation purposes; (2) enhanced monitoring of phenomena of interest for knowledge, conservation and public health purposes; (3) enhanced monitoring of diseases of interest for conservation and public health issues and to guarantee early warning (epidemiological vigilance).

All species that have never or rarely been observed stranded on a coastline and for which in-depth investigations are required are considered critical. Emblematic species are the animals already included in the strategy for monitoring contaminants in cetaceans on the French coast established by Pelagis within the framework of the MSFD (Méndez-Fernandez et al., 2019).

Phenomena of particular interest are considered to be situations corresponding to unusual mortality events and/or revealing the consequences of anthropic activities on marine mammal populations. The phenomena selected are: (1) mass strandings, (2) multiple unexplained strandings, (3) strandings suspected of being associated with noise pollution, (4) strandings suspected of being associated with acute chemical pollution, (5) lesions suspected of being of intentional anthropogenic origin and, finally, (6) strandings suspected of being related with marine biotoxins.

For diseases of priority interest, it was agreed to focus on those with known conservation or public health issues that have already had an impact in regional waters, such as avian influenza and morbilliviruses.

Each case is defined according to criteria such as the species concerned, the geographical area, the time window or the warning signs. The definition of cases must be sensitive enough to guarantee detection but also specific enough to avoid having to treat too many animals, which would not be logistically and financially feasible. For each of these cases, a procedure is established. It defines the level of examination to be carried out and a specific sampling protocol (P0) which is different for each case. Technical notes including the definition of the case, the interest of reinforcing surveillance and

the procedure to follow will be made available to all the network's correspondents.

3.1.4 Syndromic surveillance

Syndromic surveillance will consist of the continuous collection, analysis and interpretation of health indicators to ensure a rapid assessment of the impact of health risks on the health of the population monitored. The continuous signal will allow the production of a time series, *i.e.* curves, and the implementation of algorithms that detect unusual peaks in the indicators monitored, that have to be preliminary defined. This modality will achieve three objectives: (1) the anticipated detection of expected phenomena, such as an increase in bycatch mortality during the winter season or an increase in strandings in an area where a source of noise pollution is identified (e.g. during military exercises at sea); (2) the detection of unexpected phenomena, such as increased strandings at a time and in an area where this has never been described before; and finally, (3) the assessment of the impact (or lack thereof) of a phenomenon, such as the correlation between increased strandings in an area and the building of an offshore wind farm.

Only the "mortality" indicator will be systematically surveyed at short term. This will make it easier to detect mortality peaks or mortality events associated with identified phenomena. In the longer term, other indicators could be monitored continuously, such as the presence of pathogens or specific lesions as neoplasms. SyS will be carried out on all reported stranded animals. However, it will not always be possible to obtain data on all indicators for each animal as this will depend on the level of examination and analysis carried out. This monitoring modality will require a consolidated database with rapid and systematic integration of the indicators monitored and standardisation of the lexicon used to describe them. It will also require the development of adequate algorithms and a good interpretation of the results obtained.

3.2 Data standardisation

Large-scale standardised data integration has been developed to facilitate data analysis and comparison between the different information obtained from strandings. This standardisation will also facilitate the exchange of data with stranding networks in neighbouring countries. Thus, a major effort has been made to harmonise data on causes of death, lesions identified during necropsies and the results of additional analysis.

3.2.1 Standardisation of individual cause-of-death data

Individual cause of death data should be systematically included as follows: (1) initial cause of death (mandatory); (2) immediate cause of death (optional); (3) degree of certainty associated with the diagnosis of the initial cause of death (mandatory); (4) contributory causes (optional); (5) anthropogenic origin of the initial cause of death (mandatory). In addition, a commentary may accompany these data to provide details of the cause of death such as a pathogen involved.

The initial cause of death must be completed with one of the death cause categories defined (Table 3). These categories and subcategories of causes of death have been defined, similarly as it is usually done in human forensic medicine, while trying to maintain continuity with what was done previously in the network. When two or more causes of death are recorded, the veterinarian (or the biologist performing the examination) will be asked to indicate the initial cause and the immediate cause of death. For example, an animal may have suffered a predatory bite that resulted in fatal septicemia. In this case, the initial cause is indeed the bite while the immediate cause is the septicemia. The veterinarian may also indicate conditions not directly related to the cause of death but which contributed to it (contributing causes). The initial cause of death will be used for statistical analysis at the population level. Each initial cause will come with a degree of certainty on a scale of 1 to 5. The scale is the same as the one used in the Epifaune database, developed by the OFB in partnership with ADILVA (French association of directors and managers of public veterinary analysis laboratories) and Faunapath (an anatomo-pathology laboratory specialising in non-captive wildlife in Lyon). In order to facilitate the standardisation of degrees of certainty between the different levels of examination (generally stronger when the level of examination is higher), adapted definitions have been developed (Table 4). Guidelines specifying the elements that should lead to the attribution of the different degrees of certainty

TABLE 3 Categories and sub-categories of initial causes of death.

CATEGORY	SUBCATEGORY
Natural cause (resulting from a pathological condition)	• Undetermined origin • Infectious and parasitic diseases • Starvation • Lethal complications of gestation/mating • Neoplasm • Congenital anomaly • Perinatal conditions • Other (to be specified when possible)
Violent cause (traumatic or toxic)	• Undetermined origin • Bycatch • Collision • Entanglement • Predation/competition • Intentional human-inflicted injury • Noise pollution • Marine biotoxins • Chemical pollution • Ingestion of foreign objects • Other (to be specified when possible)
Accidental stranding	• Undetermined origin • Topographical • Social cohesion • Predation (active or passive) • Other (to be specified when possible)
Other cause	• Euthanasia • Other (to be specified when possible)
Undetermined cause	• Undetermined origin

TABLE 4 Definitions of level of certainty according to the level of examination (L) performed.

Level of certainty	Examination level L1 & L2: opinion on cause of death	Examination level L3 & L4: veterinary diagnosis
1	No evidence	No conclusion (Terminal cause is 'undetermined' or 'impossible')
2	Suggestive evidence	Hypothesis of diagnosis (Pathological evidence very poor or absent)
3	Evidence	Diagnosis of suspicion (Pathological evidence is present but insufficient or inconsistent)
4	Strong evidence (L2 required)	Diagnosis of well-founded suspicion (Based on consistent pathological evidence)
5	Proven cause	Definitive diagnosis (additional analysis required)

will be presented during the training sessions delivered to the correspondents in order to limit the operators biases.

Finally, it will be stated whether the initial cause of death has an anthropogenic origin or not. A commentary may be added to clarify the cause of death. For example, in the case of a natural death of infectious origin, it could be stated that it was a pneumonia compatible with a bacterial origin according to the macroscopic lesions.

3.2.2 Standardisation of necropsy data and additional analysis results

In order to facilitate analysis of the data obtained during necropsies on a population scale, the terms used to define organs and lesions, as well as the results of the complementary analysis, were standardised.

Thus, the list of organs is broken down into three levels as done in the SAGIR vademecum (Gauthier et al., 2016): matrix category, generic matrix and specific matrix. Among the matrix categories are the major categories or systems examined during marine mammal necropsies, which are as follows: whole cadaver, fetal appendages, cardiovascular system, digestive system, haemato-lymphopoietic system, respiratory system, urogenital system, oral cavity, cavities/serous membranes, muscles and tendons (active locomotor system), sense organ, skeleton (passive locomotor system), endocrine system, nervous system, integumentary system, and finally, adipose connective tissue. The generic and specific matrices make it possible to specify the organ and its possible location. For example, for the cardiovascular system it could be the heart and the myocardium for the generic and specific matrices respectively.

Similarly, lesions detected on macroscopic examination and histological analysis are broken down into three levels which are analyte category, analyte and sub-analyte. The analyte categories are: morphological abnormality, content, inflammatory, mechanical and traumatic, metabolic, proliferative and finally vascular. The analyte and sub-analyte help to specify the nature of the lesion. For example, for a vascular lesion it could be hemorrhage and petechiae for the analyte and sub-analyte respectively. The severity of the injury will be specified (mild to severe) as well as the duration of the condition (acute to chronic). Measurements may be added and the time of onset specified (*ante* or *post mortem*). Lesions may have been diagnosed by macroscopic examination or by histological analysis. Thus, the method of diagnosis will be specified.

Finally, all data from additional analysis (excluding histology) will also be integrated in a standardised way. The nature of the analysis, the method used, the presence of a positive or negative signal will be specified. The result will be broken down into three levels which are again the analyte category, the analyte and the sub-analyte. The analyte categories are numerous and include for example bacteria, viruses, parasites, fungi, toxins or contaminants. The analyte and sub-analyte will help to specify the agent of interest. In the case of biological agents, these are the genus and species respectively. Finally, the veterinarian should specify whether the agent in question is considered to have caused disorders in the animal's lifetime.

4 Discussion

The health monitoring of marine mammals as presented here is an ambitious project requiring as many stranding data as possible. Pelagis' first ambition was to improve its means of expertise in order to conclude with more precision and certainty on the causes of mortality. The development of these means was the opportunity to reinforce the global health monitoring of marine mammal populations. This strategy will allow to improve the knowledge of the health status of these species subjected to numerous pressures by following the evolution in time and space of diseases associated with conservation and public health issues while ensuring the early detection of emerging and/or zoonotic diseases of importance. It will also allow a better assessment of the consequences of anthropic activities on these animal populations and on the environment.

The high number of strandings in metropolitan France, combined with logistical, financial and human resources, imply that the highest level of examinations cannot be implemented on every stranded marine mammal. Thus, a strategy with different surveillance modalities has been established, allowing us to choose the individuals on which the most in-depth investigations will be carried out, while representing at best the stranded marine mammal population. The strategy, based on four surveillance modalities which are general event-based surveillance, programmed surveillance, specific event-based surveillance and syndromic surveillance should imply many evolutions. Among these, the main ones are the dissemination of expertise means and their

appropriation by the network actors, the ability to meet the sampling plan and the appropriation of the P3 sampling protocol within the framework of the programmed surveillance and the integration of standardised data (standardized samples and analysis), which imply a significant reorganisation of the database structure.

The systematic performance of standardized analyses within the framework of the PS will make it possible to carry out descriptive epidemiology by identifying the health problems of populations and by measuring them in time and space. In addition to being able to infer the results obtained to a reference population, the laboratory analyses that complete the necropsy allow the cause of death to be investigated with greater precision.

Various criteria were considered in order to define the reference population for the sampling plan to conduct the PS. Emblematic Species already included in the strategy for monitoring contaminants in cetaceans (bottlenose dolphin for the 3 metropolitan seaboards, common dolphin for the Atlantic coast, harbour porpoise for the English Channel coast and stripped dolphin for the Mediterranean coast) on the French coast established by Pelagis within the framework of the MSFD were considered as a priority (Méndez-Fernandez et al., 2019). Moreover, to date, very few studies have been conducted on the pathogens circulating in marine mammal populations in France, not allowing species to be targeted according to their health vulnerability, with the exception of the striped dolphin, which has been the victim of mass mortalities caused by morbillivirus on several occasions in regional waters (Dhermain et al., 1995; Keck et al., 2010; Van Bressem et al., 2014) and harbour seals which have been victims of Avian Influenza in the North Sea (Bodewes et al., 2015). These two species were therefore also considered as a priority. The lack of general data at this stage has therefore led to the selection of the most frequently encountered stranded marine mammal species as reference populations in the first instance, while ensuring that the previously mentioned priority species are included. This will allow us to obtain initial data on the circulation of pathogens in these populations as well as on their health impact, and to subsequently make more specific choices concerning the marine mammal species and pathogens monitored. It is expected that after a period of one or two years, other strategic choices will be made. For example, instead of conducting the PS on a representative sample of the entire stranded population, the priority could be shifted to emblematic species. However, the distribution of logistical means and expertise on the territory did not allow to make this choice at this stage: for example, to be interested in the striped dolphins in the same way as the common dolphins would imply making a very important logistical effort in the Mediterranean coast. Thus, according to the current sampling plan, some species-seaboard pairs are under-represented, or even unrepresented when they constitute less than 0.5%. It will therefore be difficult or impossible to obtain valid data for these species, such as the long-finned pilot whale, which will be only marginally represented in the plan. The same applies to the maritime frontage. With less than ten animals in the sampling plan for the Mediterranean the information obtained will be very limited. It is also important to remember that current knowledge does not allow us to estimate the representativeness of the living marine mammal population based on strandings, which also implies a major limitation. Furthermore, the sampling plan is confronted with major sampling biases that must be considered when analyzing the various data obtained. Indeed, a series of non-standardised filters related to detectability, reporting, access to carcasses, DCC, human and logistical response capacities make these biases unavoidable and must therefore be considered when analyzing and interpreting the data.

Morevover, as these are mobile species that should be considered on a basin or regional scale, collaborations with neighbouring countries where strandings of animals from the same populations as those occurring on our coasts will be necessary. Although necropsy and common sampling protocols have already been proposed on a European scale, the situation is different for routine analysis (IJsseldijk et al., 2020a). Thus, a minimum of common routine analysis should be considered in order to obtain more global and representative data of these animal populations and to ensure large-scale health surveillance in a One Health context. The agreements of ASCOBANS and ACCOBAMS as well as the ICES and OSPAR working groups and the European Cetacean Society should be the framework for discussions and exchanges on practices with other European countries in order to harmonise not only necropsies and tissue sampling (Jauniaux et al., 2019; IJsseldijk et al., 2020a) but also the complementary analysis carried out by the laboratories as initiated at the pathology workshop of the European Cetacean Society in 1991 (Kuiken and García Hartmann, 1991).

The choices concerning standardised routine analysis within the framework of P3 and therefore of programmed surveillance were made according to several criteria: the improvement of knowledge and the interests for conservation and public health in relation to the financial cost and technical and logistical feasibility (easy access to laboratory techniques).The post mortem investigation and tissue sampling protocol already established under the auspices of ASCOBANS (Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Seas) and ACCOBAMS (*Accord sur la Conservation des Cétacés de la Mer Noire, de la Méditerranée et de la zone Atlantique adjacente*) (IJsseldijk et al., 2020a), has guided our choices as well as the most frequently analysis performed in foreign European networks (Prenger-Berninghoff et al., 2008; van de Velde et al., 2016; Kershaw et al., 2017; Sonne et al., 2020; Stokholm et al., 2023). Investigations for morbilliviruses, influenza viruses and *Brucella* sp. will be sought for their known impact on marine mammal populations for the first two and for their zoonotic character for the last two. Morbilliviruses have indeed been the cause of mass mortalities on several occasions in regional waters, in the Mediterranean and in the North and Baltic Seas (Dhermain et al., 1995; Rijks et al., 2008; Keck et al., 2010; Duignan et al., 2014; Van Bressem et al., 2014). For influenza, a mass mortality event has also occurred in the North Sea (Bodewes et al., 2015). Furthermore, the risk for public health is not negligible as the high reassortment potential of this virus means that it could become more virulent and pathogenic, both for marine mammals and humans (Anthony et al., 2012; Fereidouni et al., 2016; Van den Brand et al., 2016). Similarly, brucellosis of marine origin, in addition to affecting reproduction, has repeatedly caused

infections in humans and is most likely under-diagnosed (Brew et al., 1999; Jouffroy, 2020). Moreover, herpesviruses will be investigated as various studies have shown that these viruses circulate widely in marine mammal populations and that the clinical pictures, although variable, can lead to death (Duignan et al., 2018). Finally, mycotic agents will be looked for in their entirety as they are considered a major emerging cause of mortality in both humans and marine mammals (Reidarson et al., 2018). Other pathogens for which the risks are less known or for which massive mortality events have occurred but in distant waters have not been retained at this stage. They will nevertheless be the subject of reflection by a working group on the prioritisation of health hazards in marine mammals and may at any time be included in the P3 protocol or in the case of SES. In addition, future collaboration with neighbouring countries may identify other risks to be monitored.

The results of analysis carried out on carcasses, although they are of DCC 1 or 2, should be interpreted with caution, particularly for bacteriological analysis, due to possible *post-mortem* contamination (Palmiere et al., 2016). Also, it should always be considered that pathogen testing on carcasses allows us to know if the animal was infected at the time of death but not if the animal was infected earlier and then cured. Therefore, it does not give an overall picture of the circulation in the population. For this, it would be desirable to obtain seroprevalences as it has been carried out for avian influenza in seals (Bodewes et al., 2015; Measures and Fouchier, 2021).

Epidemiological vigilance will be ensured by the different modalities, in particular for cases managed with specific event-based surveillance. The example of the major avian influenza outbreak in Europe in 2022 (Adlhoch et al., 2022) reminds us of the importance of detecting potential outbreaks in marine mammal populations. Epidemiological vigilance will be constrained by certain limitations such as the DCC of the carcass, which will have a strong impact on the ability to obtain quality information. The identification of cases subject to specific event-based surveillance based on spontaneous reporting by correspondents implies that they are well informed and that case definitions are simple, sensitive and specific enough. In addition, case management under SES requires the availability of network correspondents trained in the level of examination and sampling protocol required for the case but also the involvement of laboratories performing the defined analysis. Although the volume of animals covered by this surveillance modality can be estimated, it is important to note that it is impossible to know precisely the number, nature and location of data that will be collected.

Beyond the defined strategy, *ad hoc* surveys may be carried out to answer specific questions from research projects or to respond to questions related to the epidemiological context, as could have been done by looking at the susceptibility of marine mammals to SARS-CoV-2 in marine mammals during the epidemic that Europe experienced, as it has been done in Italian waters for example (Audino et al., 2021).

Many aspects of the strategy are based on data obtained over the last five years for which all the information is already available (2016 to 2020). This arbitrary choice of a relatively short period was made to consider population shifts and changes in the environment (environmental parameters, fishing practices, construction of offshore parks…) and to try to be as close as possible to what might happen in the coming year. The time windows of the MSFD could possibly be used as early as 2025 (beginning of the third MSFD cycle) in order to harmonise the strategies.

Generally speaking, the strategy for strengthening health surveillance developed by Pelagis provides a framework that allows all the actors in the NSN to be informed of the approach followed and to understand its ins and outs. Thus, the effort of training (a 3 days initial training is required to incorporate the NSN, and retraining every five years at least, concurrently with the evolution of protocols) and informing correspondents is essential to guarantee their support and investment in the project and to limit operator bias harmonising practices. It is also important to continue to train new correspondents at the various levels, as the more numerous they are, the greater the number of animals evaluated.

The overall cost of this strategy, implemented in January 2023, is significant mainly due to the laboratory analysis carried out within the framework of the PS, which are added to the logistical costs of managing strandings. The discussions that will be conducted by the working group on the prioritisation of health hazards should make it possible to reach a compromise between the benefits of these analysis and the associated cost in the next two years.

Although there are many constraints to strengthening health monitoring, the structure of the NSN is very similar to that of an epidemiological surveillance network, which makes it a major strength. Moreover, the network has already proven its capacity to detect almost all strandings and to collect data on a large scale in space and time.

Similarly, health monitoring should focus on live marine mammals too. This mainly concerns pinnipeds, including those entering care centres before being released, but also live cetacean strandings. Finally, marine mammal populations in French overseas waters that face different problems to those in metropolitan France should also be subject to health monitoring.

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

The main work was carried out by SW under the guidance of FC, and with the main support of EM. All authors contributed to the article and approved the submitted version.

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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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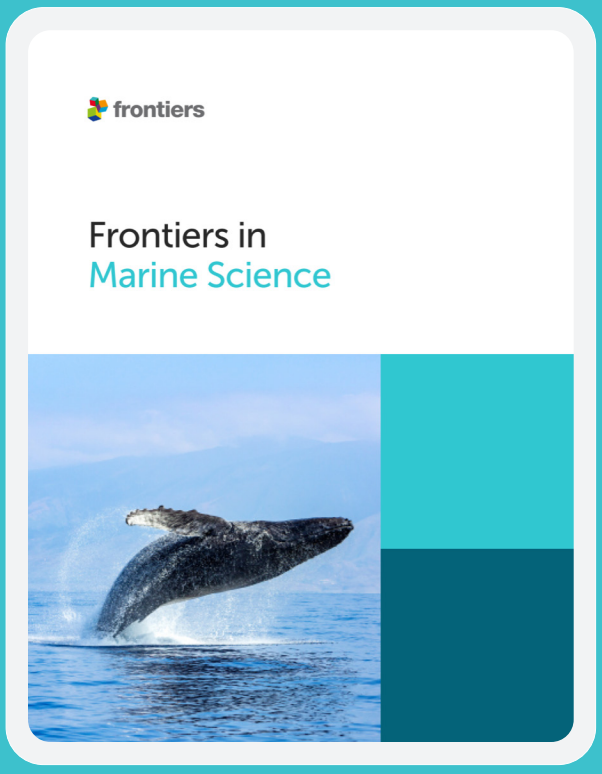

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