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High-Resolution Longitudinal eDNA Metabarcoding and Morphological Tracking of Planktonic Threats to Salmon Aquaculture

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ABSTRACT

Salmonid aquaculture, a major component of the Northern European, North American, and Chilean coastal economies, is under threat from challenges to gill health, many of which originate from plankton communities. A first step toward mitigating losses is to characterize the biological drivers of poor gill health. Numerous planktonic taxa have been implicated, including toxic and siliceous microalgae, hydrozoans, and scyphozoans; however, rigorous longitudinal surveys of plankton diversity and gill health have been lacking. In the current study, we present and assess an exhaustive identification approach combining both morphological and molecular methods together with robust statistical models to identify the planktonic drivers of proliferative gill disease (PGD) and fish mortality. We undertook longitudinal evaluation at two marine aquaculture facilities on the west coast of Scotland using daily data collected during the 2021 growing season (March–October). Examining these two different sites, one sheltered and one exposed to the open sea, we identified potentially new, important, and unexpected planktonic drivers of PGD and mortality (e.g., doliolids and appendicularians) and confirmed the significance of some established threats (e.g., hydrozoans and diatoms). We also explored delayed or "lagged" effects of plankton abundances on gill health and undertook a comparison of environmental DNA (eDNA) metabarcoding and microscopy in their ability to identify and quantify planktonic species. Our data highlight the diversity of planktonic threats to salmonid aquaculture as well as the importance of using both molecular and morphological approaches to detect these. There is now an urgent need to expand systematic longitudinal molecular and morphological approaches across multiple sites and over multiple years. The resultant catalogue of main biological drivers will enable early warning systems, new treatments, and, ultimately, a sustainable platform for future salmonid aquaculture in the marine environment.

María Algueró-Muñiz and Sofie Spatharis are co-first authors.

1 | Introduction

Aquaculture is the fastest-growing food production sector globally and has undergone rapid expansion and diversification in recent decades (Naylor et al. 2021). Current marine production of finfish accounts for over 8.3 million tonnes globally (FAO 2022) and is dominated by four species: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), European seabass (*Dicentrarchus labrax*), and gilt-head seabream (*Sparus aurata*) (Naylor et al. 2021). Most marine finfish are grown in floating cages anchored to the seabed in the coastal environment. Unlike their wild progenitors, fish in net pens are contained within the net volume, and while they do show avoidance behavioral responses to water quality changes, penned salmon have limited ability to avoid significant phytoplankton bloom or jellyfish swarm events. Consequently, marine finfish aquaculture is heavily dependent on the quality of the local biotic and abiotic environment to sustain survival and optimal growth. Climate change is affecting aquaculture worldwide at local and global scales (Maulu et al. 2021). Coastal environments are experiencing the simultaneous impacts of multiple anthropogenic stressors (Martinez and Rusch 2021) such as warming and eutrophication; sea surface is warming at an unprecedented rate, meanwhile terrestrial nutrient run-off from domestic and agricultural activities is driving coastal phytoplankton blooms (Dai et al. 2023). The resultant combination of increasing phytoplankton and zooplankton productivity, alongside accelerated parasite development and lowered dissolved oxygen (DO) (Dalvin et al. 2020; Guerrero et al. 2018; Jones and Price 2022), represents a significant and growing challenge to aquaculture in coastal environments globally.

In recent years, poor gill health has emerged as a major contributor to reduced farmed finfish survival at sea, especially in Atlantic salmon (Boerlage et al. 2020; Herrero et al. 2022). Gill health-related losses in the Scottish salmon aquaculture have been steadily increasing particularly in the late summer months, for example, 1.57% in September 2018 compared to 4.65% in September 2022 (Salmon Scotland 2022). The gill plays several vital roles in teleost physiology: a mucosal barrier for the immune system, gas exchange, osmoregulation, excretion of nitrogenous waste, and hormone production (Foyle et al. 2020). The gill surface is in continuous contact with the marine environment and as such continually exposed to potential biotic and abiotic stressors. Gill health challenges are multifactorial as several transmissible parasites, viruses, and bacteria are implicated (e.g., Rodger 2007). Nematocysts—stinging organelles associated with cnidarian epithelial cells—are also thought to drive gill damage and inflammation (Kintner and Brierley 2019). Phytoplankton species may also have a role, either via toxin production or, in the case of siliceous diatoms, as direct damage to the gill via penetration of the siliceous spines in gill tissue (Bell 1943; Østevik et al. 2022). Biological stressors on salmon gill health may synergistically interact to drive cumulative damage to gill tissue. For example, initial exposure to hydrozoans can result in secondary parasitic infection (e.g., Kintner and Brierley 2019), although experimental challenge trials have been inconclusive in proving the link between hydrozoan exposure and subsequent *Neoparamoeba perurans* infection severity (Bloecher et al. 2018).

Pathological responses in salmon gills encompass amoebic gill disease (AGD), proliferative gill disease (PGD), and other gill damage that fall under the umbrella "syndrome" of complex gill disorder (CGD) (Noguera and Marcos Lopez 2019). CGD includes a wide range of clinical gill disease presentations generally occurring from summer to late autumn on marine Atlantic salmon farms and relates to both AGD and PGD, which can cause gill necrosis, respiratory distress, and, ultimately, fish death. For most PGD cases in farmed Atlantic salmon, no specific pathogen or other harmful agent can be incriminated as the causative agent. Over 40 zoo- and phytoplankton species are suspected to be involved (Boerlage et al. 2020). Many salmon producers monitor the water column for these species daily to detect blooms in a timely fashion and instigate the limited mitigation measures at their disposal (e.g., feed reduction, plankton skirts, and bubble nets).

Currently, the primary mode of plankton monitoring on fish farms involves morphological identification via light transmission and binocular microscopy (for phyto- and zooplankton, respectively) in mostly single time-point water samples taken throughout the water column. Accurate taxonomic identification of morphologically similar species is challenging and time-consuming and is subject to human error, with significant taxonomic expertise required in-house (Deagle et al. 2018). Effective disease mitigation relies on early intervention following detection above arbitrary thresholds, especially for algal and cnidarian blooms (Engehagen et al. 2021). To achieve this, planktonic monitoring must be rapid, accurate, quantitative, and encompass multiple taxonomic levels including metazoans and protists. Furthermore, many of the planktonic drivers of gill pathology in salmonid aquaculture are unknown, and/or their association with poor gill health is circumstantial and lacks a robust statistical framework.

In the current study, we assessed an "exhaustive" approach to plankton identification combining both morphological and molecular (environmental DNA a.k.a. eDNA metabarcoding), in combination with robust statistical models, to identify the planktonic drivers of CGD and mortality in salmonid aquaculture. To achieve this, we undertook longitudinal molecular and microscopic evaluation on a daily basis during the production period (March–October 2021) at two marine aquaculture facilities on the west coast of Scotland. Examining these two sites, one sheltered and one exposed to the open sea, we identify new and unexpected planktonic drivers of CGD and mortality and confirm the importance of some established threats. We also explore delayed or "lagged" effects of planktonic abundances on gill health as well as undertake a comparison of eDNA metabarcoding and microscopy in their ability to identify planktonic species and estimate their abundance.

2 | Methods

2.1 | Sites and Sample Size

Between March and October 2021, we monitored two sites on the NW coast of Scotland, UK: a sheltered site with a large freshwater input was monitored for 223 sampling days, while an exposed open water site provided us with 191 sampling days.

This daily monitoring included phytoplankton and zooplankton surveys, as well as eDNA sampling. Moreover, matching our sampling period, our collaborators on the farms provided mortality data (interpolated z-score data), PGD and AGD scores (scaled from one to five for increasing severance, see Noguera and Marcos Lopez 2019, for details), environmental data related to temperature, oxygen levels, salinity, and visibility in the water adjacent to the cages, and treatments applied to fish during our study. We then used best-fitting models to determine the strongest planktonic predictors of diminished fish health. Figure 1 represents approach, outputs, and applicability of the methodological framework of our study. Plankton symbol attribution: ian.umces.edu/media-library.

2.2 | Plankton Sampling and Identification via Microscopy

Species were identified by both classic (morphological) and molecular approaches. While the classic approach was thought to offer a more accurate representation of species abundance, we considered that the molecular approach might offer a more exhaustive species list—at the level of the operational taxonomic unit (OTU)—as it can capture species that are rare and/or small and thus undetected by microscopy. Samples were analyzed morphologically using conventional microscopy techniques and genetically via metabarcoding of a fragment of the cytochrome oxidase I (COI) gene (see next section).

Phytoplankton was sampled at 5 m depth with a Van Dorn bottle and 250 mL of seawater was immediately preserved with acidic Lugol's iodine solution (5%). Samples were preserved in dark glass bottles in a dark cool place for 3–6 weeks before microscopic analysis. Samples were then filtered through nitrate cellulose membranes of 0.45 μm mesh (Fournier 1978) followed by transillumination of the filter with immersion oil and observation under a Zeiss Axiolab upright microscope. This approach was adopted due to the observed underestimation of the smaller fraction of phytoplankton (<20 μm) by the inverted microscope method (Utermöhl 1958). Taxa were identified in their majority to species level, and cell counts were expressed as number of cells per liter.

Zooplankton was sampled in the vicinity of the salmon nets by vertical net hauls, and sampling depth was restricted to 10 m to mimic the depth of the fish net pens. During the experimental period, one net haul per site was collected. In the exposed site, sampling was conducted with an Apstein net (55 μm mesh size, 25 cm diameter), while in the sheltered site we used a plankton net (55 μm mesh size, 30 cm diameter), both equipped with a closed cod end, every net haul consisting of total filtered volumes of 491 and 707 L, respectively. Samples were then rinsed with seawater prefiltered through 50 μm mesh, collected in containers, and preserved in 4% formaldehyde buffered with sodium tetraborate. During analysis, organisms were sorted using a stereomicroscope (Leica S9i) and classified to the lowest possible taxonomic level.

Initial target zooplankton taxa were represented by salmon sea lice and hydromedusae, based on literature references and direct communication with the farms. Copepod nauplii from different species were pooled together, except for sea lice species *Lepeophtheirus salmonis*, which were monitored independently. Every sample was sieved through 50 μm mesh, rinsed with tap water, and poured into a calibrated beaker, where organisms were well mixed before subsampling three aliquots with a Hensen Stempel pipette (Harris et al. 2000), representing a minimum of 12% volume of the sample. Morphological quantification was restricted to 12% of volume, whereas the remaining sample volume was monitored for the taxa not recorded in the aliquots to record diversity, with a special focus on the target species.

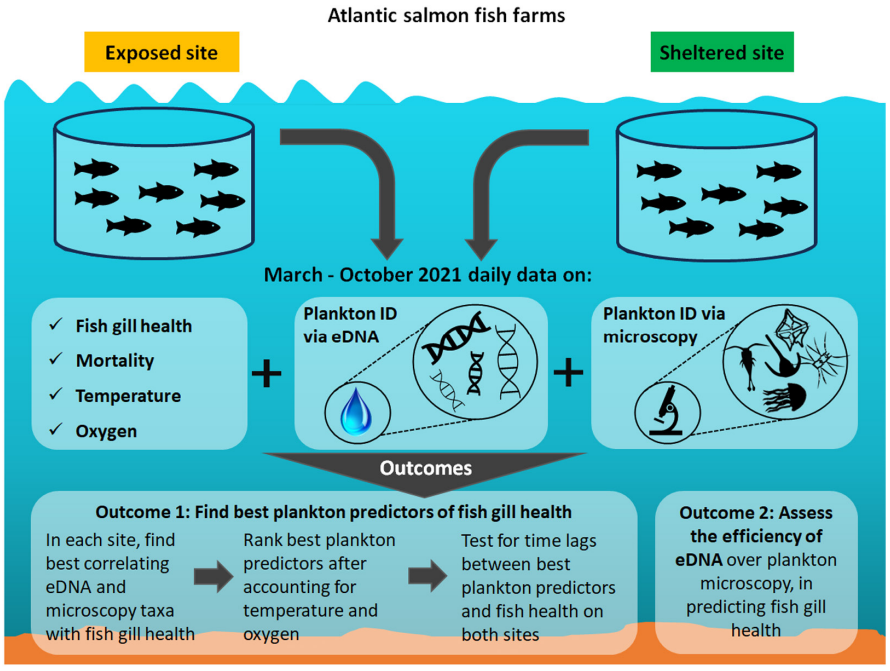

FIGURE 1 | Approach, outputs, and applicability of the methodological framework of our study. Plankton symbol attribution: ian.Umces.edu/media-library.

2.3 | Environment DNA Sampling, Metabarcoding, and Sequencing

Water from 5 m depth was sampled by using a Niskin bottle (Sheltered site) or a Van Dorne (Exposed site), and 500 mL of seawater was filtered through a sterile 0.2 μM filter (Sterivex, Merck) in technical duplicate on-site by aquaculture staff using a modified Spear & Jackson 5 L pump sprayer. The sprayer was rinsed thoroughly in seawater each day and 1 L of sampled water was used to flush the system prior to connecting the filter unit. Filters were pumped dry, sealed into sterile 50 mL centrifuge tubes including c.20 g of silica bead desiccant, and stored at room temperature prior to transfer to the molecular biology laboratory in weekly batches via courier. DNA extraction was achieved using a Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocols with a minor modification. As a first step, 500 μL of lysis buffer was added to each filter unit, and the unit was gently shaken overnight at 56°C on a rotary wheel prior to transferring the lysate to the spin-column, washing, and elution into a 2 mL centrifuge tube as in Turon et al. (2022). Negative extraction controls were included in the lab following the same protocol. The partial COI Leray-XT fragment (313 bp) was amplified from these metagenomic DNA samples using the mlCOIintF-XT/jgHCO2198 (Leray et al. 2013) primer pair (Wangensteen et al. 2018), primer sequences used were GGWACWGGWTGAACWGTWTAYCCYCC (mlCOIintF) and TAIACYTCIGGRTGICCRAARAAYCA (jgHCO2198). Leray primers included spacers to increase complexity, as well as dual index barcodes to assist with the multiplex. PCR products were bead-purified prior to PCR-based tagging to achieve high-level (600+) multiplex using dual index 96 barcoded primers, alongside Illumina P5 and P7 adaptor and Nextera sequencing primer binding sites. The final library was gel purified, quantified, and submitted for 250 bp paired-end sequencing on an Illumina NovaSeq 6000 instrument at Novogene PLC.

Custom demultiplexing of internal and external barcodes was undertaken using a combination of flexbar (Dodt et al. 2012; Renaud et al. 2015) and deML (Renaud et al. 2015). Operational taxonomic units (OTUs) were identified and assigned to taxonomy from demultiplexed samples using the MJOLNIR v1 pipeline (https://github.com/uit-metabarcoding/MJOLNIR) as in Turon et al. (2022). MJOLNIR implements a variety of programs from the OBITOOLS package (Boyer et al. 2016) for sequence preprocessing and taxonomic assignment as well as VSEARCH (Rognes et al. 2016) and SWARM (Mahé et al. 2022) for chimera detection and OTU clustering, respectively. The median read depth per sample was 220,000. Fourteen negative extraction controls were amplified and sequenced. Appreciable contamination (c.20 K reads) was noted only in one negative control. OTU taxonomic identities were passed directly to correlation analyses with biotic and abiotic variables, as well as with microscopy data. Data are available on the NCBI Short Read Archive (SRA) under bioproject PRJNA1122127.

2.4 | Data Analysis

Plankton taxa identified by eDNA metabarcoding were aggregated at the genus level unless there was only one species representing the genus in which case the name of the species was retained (e.g., *Lizzia blondina*). In microscopy data, the name of the genus was used when higher resolution could not be achieved with microscopy (e.g., *Chaetoceros*, *Pseudo-nitzschia*). We also created higher taxonomic level aggregations, for example, phyla or classes depending on the purposes of the analysis and data visualization. For the purposes of statistical modeling, we transformed our eDNA metabarcoding data to compositional data using the R package "compositions" v.2.0-8 and the function "acomp" (van den Boogaart and Tolosana-Delgado 2008) which helps transform the data into compositional data in cases such as ours where "the total amount is meaningless or the individual amounts are part of a whole (in equal units) and the data should be analyzed in a relative geometry." We apply this transformation in lines 73–76 of our revised R code which we submitted with the revised manuscript. Our inference from the modeling approach and taxa correlations remained the same. After transformation, we excluded from the OTU-read dataset all reads that corresponded to fish, birds, mammals, and terrestrial plants.

For multivariate analysis of microscopy and eDNA metabarcoding species data, we determined between-sample similarities using Jaccard's distance and visualized these with multidimensional scaling ordination using the "vegan" R package v.2.6-4 (Oksanen et al. 2022). For this analysis, we used non-interpolated presence-absence data at the highest resolution level for both eDNA metabarcoding data and microscopy plankton data.

For modeling purposes, data gaps along the timeline were filled, separately within each site, using linear interpolation with the function na.approx and the R package zoo v1.8-12 (Zeileis and Grothendieck 2005). We followed the rule that only allows interpolation and no extrapolation beyond the available values of a variable at the edges of the timeline. Specifically, although most of our data (e.g., plankton species abundances, DO, Temperature) were available on a daily basis, the PGD score data were available on a weekly basis at both sites whereas the mortality of the sheltered site was also based on z-score values of interpolated weekly data. Linear interpolation was also used to fill missing values in the plankton timeline, for instance when samples could not be collected due to adverse weather conditions.

To test for potential positive associations of plankton-borne organisms with the incidence of fish PGD and mortality, we applied a three-step conservative model selection process of plankton predictors. As a first step, for each of the two sites separately, we selected all species that showed a >0.4 Spearman correlation with either PGD or mortality. As a second step, within each site separately, we fitted a linear model with PGD or mortality as response variables and as explanatory variables the highly correlated species that were selected from step 1. In addition, here we also accounted for temperature and oxygen as covariates in the model to account for the direct effect they can exert on fish health. Specifically, dissolved oxygen concentration is a known predictor of fish welfare (Remen et al. 2016) and more recently temperature has also been reported as a direct predictor of gill pathology (Herrero et al. 2022) and has been found to alter the bacterial microbiome in fish gills that are associated with disease (Ghosh et al. 2022). To test the individual effect of each species on

PGD or mortality, after accounting for temperature, oxygen, and the other species present, we used the *glmm.hp* R package v.0.1.2 (Lai et al. 2022). The *glmm.hp* function determines the relative importance of collinear predictors by partitioning variation explained by each covariate into unique and average shared and is recommended for datasets with multiple collinear variables such as ours. The sum of these two components (unique and average shared variation) was used to express the percentage of the individual contribution of each predictor species in our model. The selected species from step 1 were added to the model with forward selection starting from the highly correlated and proceeding to the weakly correlated while discarding those with <3% individual effect on the total variation in either PGD or mortality. As a third step, we determined whether the effect of the selected species depended on the site exposure level. To achieve this, we merged the data from both sites and fitted a PGD and a mortality model using as explanatory terms the temperature, oxygen, and the factor exposure level (exposed/sheltered) as well as all the species selected from step 2 and their interaction with exposure level. All explanatory covariates were scaled for inclusion in the models. This three-step process was then repeated to test the effect of lagged PGD and mortality data by 2, 5, and 10 days behind the plankton species data to establish any lag effects on fish health. The lagging of data was performed on the fish condition data using the package lubridate v.1.9.3 (Grolemund and Wickham 2011) and subsequently, this lagged dataset was merged with the non-lagged species-abundance dataset. All analysis was carried out in R v.4.3.1. (R Core Team 2022).

3 | Results

Fish mortality within each of the two aquaculture sites presented strong temporal variability with notable increases observed from June onward. In the exposed site, mortality peaked in late June and September whereas in the sheltered site, the highest mortalities were recorded in August and September 2021 (Figure 2). PGD scores also increased in late summer and were overall higher in the sheltered site. The total abundance of phytoplankton and zooplankton presented multiple peaks throughout the study period, with higher abundances during the summer months particularly in the sheltered site.

The eDNA metabarcoding approach assigned 9577 OTUs. Upon aggregation of OTUs at the genus level and exclusion of non-relevant taxa (e.g., mammal, fish, amphibian, and avian DNA), the total number of plankton genera was 447. Plankton taxa identified by microscopy included 185 taxa at the species level (for phytoplankton) and genus or class level (for zooplankton), and WoRMS database was used for species names validation (WoRMS Editorial Board 2024). The relative abundance (as relative reads in the sample) of dominant phyla identified by eDNA metabarcoding presented temporal and geographical

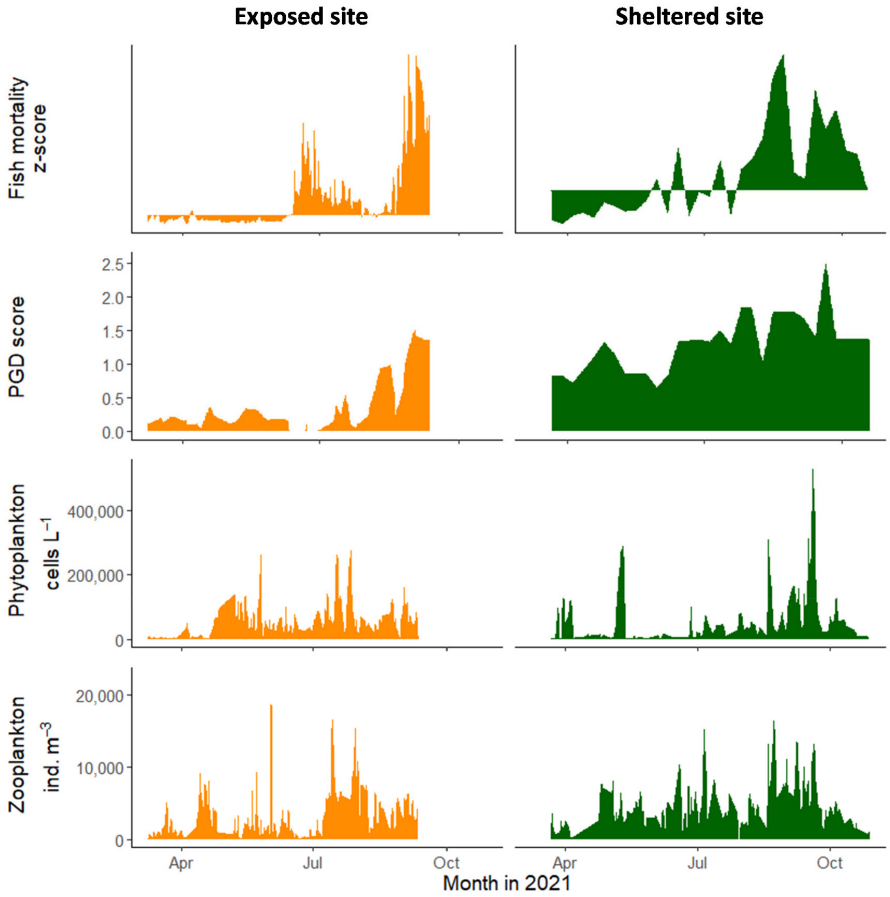

FIGURE 2 | Dynamics of fish condition variables and total phytoplankton and zooplankton abundance—via microscopy—during the study period (March–October 2021). Trends are shown for an exposed to the open sea versus a sheltered aquaculture site. Fish mortality is based on interpolated z-scores that were calculated separately for each site.

differences (Figure 3A). The exposed site was characterized by a large number of unassigned OTUs, and the top six phyla in decreasing abundance were Unassigned > Discosea > Ascomycota > Bacillariophyta > Cnidaria > Rotifera (Figure 3B). For the sheltered site, the top six phyla in decreasing abundance were Discosea > Unassigned > Rotifera > Bacillariophyta > Cnidaria > Ascomycota.

Plankton species composition was different between the exposed and sheltered sites, and the difference was more distinct when eDNA metabarcoding data were used (Figure 4 left panels). Temperature was a driver of compositional patterns irrespective of site, but this pattern was more clear when using microscopy data (Figure 4 middle panels). This pattern was mainly driven by plankton taxa such as *Rhizosolenia setigera*, *Chaetoceros*, *Dictyocha*, *Lizzia blondina*, and *Oikopleura dioica* which showed increased abundances from June onward. Very few taxa showed increased abundance throughout the production period such as the copepods and the diatom *Pseudo-nitzschia* (see also Figure 6). Compositional patterns are less associated with oxygen levels and that is consistent with both types of data (microscopy and metabarcoding).

Temperature increase and dissolved oxygen decrease were significantly and strongly associated with fish mortality and PGD prevalence at both sites (Figure 5). Multiple phytoplankton and zooplankton taxa were positively associated with PGD and mortality, and this effect was often dependent on the site. For example, doliolids were only present in the exposed site, where they appeared to be associated with both mortality and PGD, whereas the appendicularia *Oikopleura dioica* was present on both sites and its effect on PGD was site-dependent (Table 1).

Ceratium, doliolids, *O. dioica*, and ophiuroid larvae (including *Ophiothrix* sp. and *Ophiura* sp.; grouped as "ophiura larvae") were associated with both PGD and mortality, whereas some species were uniquely associated with either PGD or mortality. Specifically, *Chaetoceros*, *Ostreococcus*, *Cylindrotheca*, and sea urchin larvae were uniquely associated with PGD, whereas *Attheya*, *Prorocentrum micans*, *Pseudo-nitzschia*, *Frustulia*, and *Rathkea octopunctata* were uniquely associated with fish mortality (Table 1). Some plankton species appeared associated with PGD and mortality only when lags were included at 2, 5, or 10 days, reflecting a delayed impact. Specifically, *Cylindrotheca* was associated with PGD with a 5- and a 10-day lag (Table 1), and *Attheya*, *Pseudo-nitzschia*, and *R. octopunctata* were associated with mortality when the latter was lagged by more than 2 days.

The abundance of plankton species which showed a significant association with fish mortality and PGD showed strong temporal variation throughout the study period from March to October 2021 with most species increasing in abundance over the summer months (Figure 6). An exception to this summer

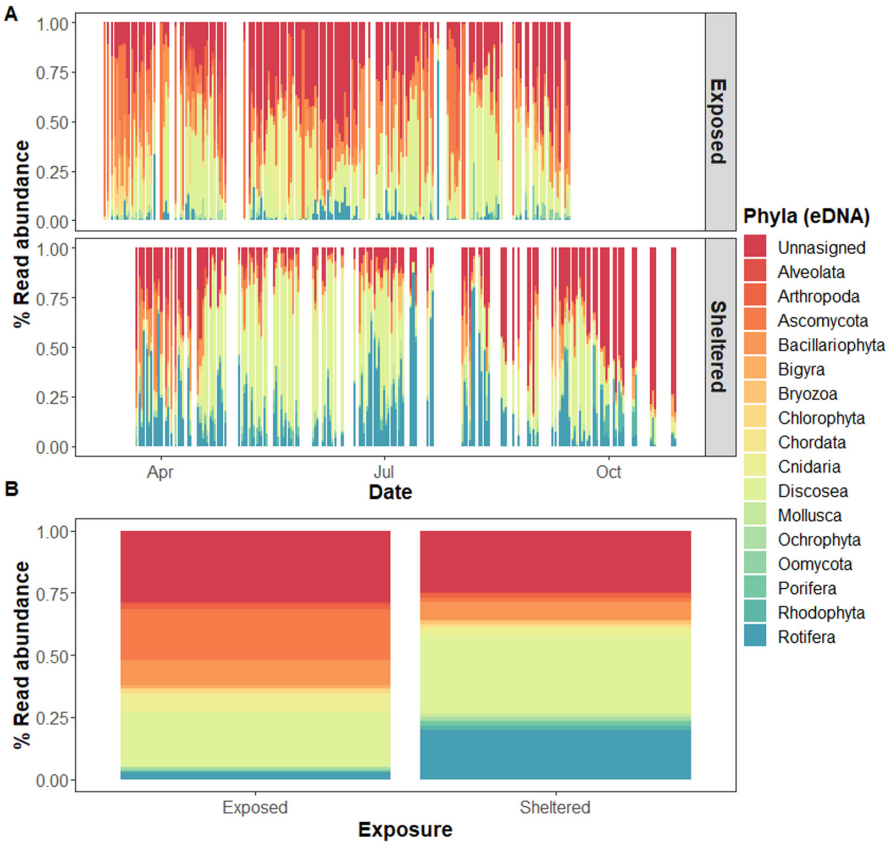

FIGURE 3 | Classification of relative read abundances of Operational Taxonomic Units (OTUs) into the most abundant phyla identified via eDNA metabarcoding. (A) shows temporal patterns across the study period (March–October 2021) and the two aquaculture sites (exposed and sheltered) while (B) presents the same information but aggregated across all daily samples within each site. Taxonomic identities of OTUs were manually curated using BLASTn, and only the Phyla contributing to >1.5% of reads were included in the graph. OTUs with no known taxonomic classification are shown as "Unassigned."

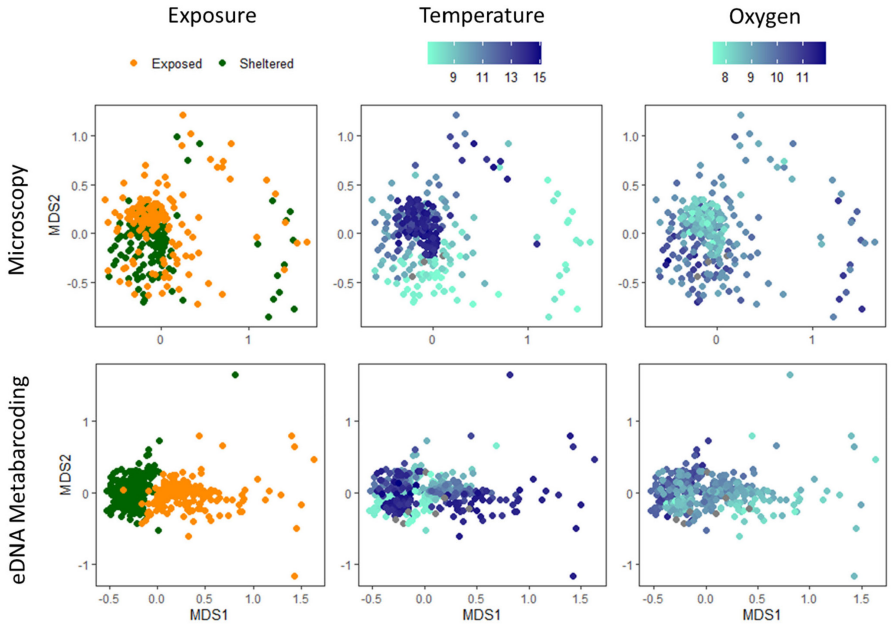

FIGURE 4 | Multi-dimensional scaling ordination showing the relative similarity of plankton composition across our daily samples from the two sites of different exposure level. Analysis was based on the Bray–Curtis similarity index calculated on non-interpolated, species presence-absence microscopy data and genus presence-absence eDNA metabarcoding data. Sample coloration is shown with respect to the site's exposure level (left panels), temperature (middle panels), and oxygen level (right panels).

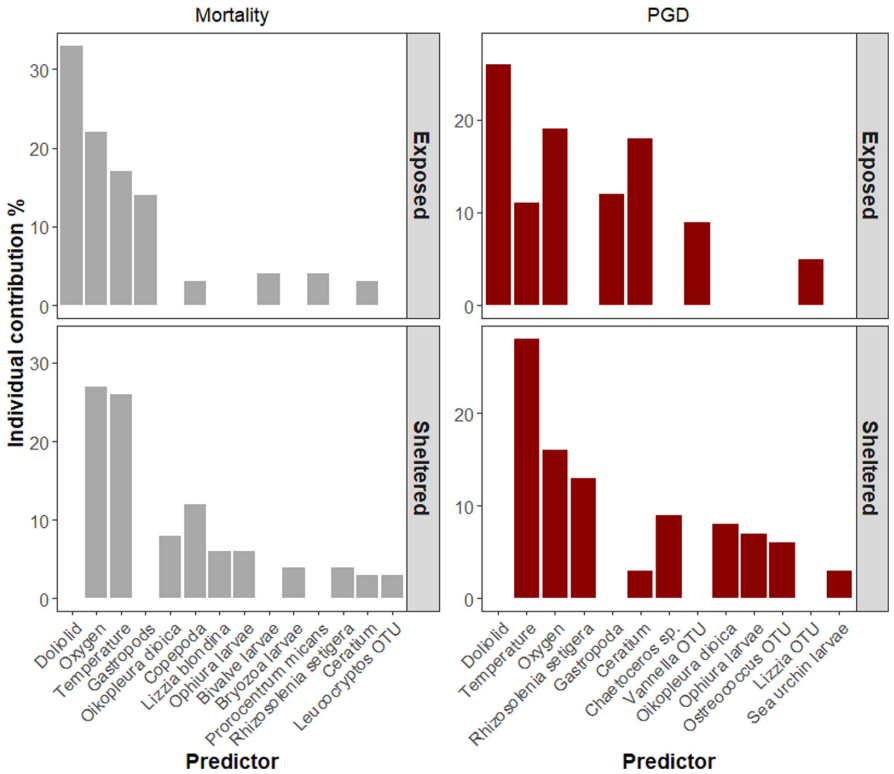

FIGURE 5 | Ranking of the relative importance of predictor variables for fish PGD and mortality within sites. The ranking is conservative and is based on a preselection of predictors whereby those correlated with Spearman $R^2 < 0.4$ with either PGD or mortality were excluded. The model included the top predictors which have an individual contribution of >3% to the overall shared variation in PGD and mortality.

increase were copepod, gastropod larvae, and bivalve larvae, whose abundance was high throughout the period on both sites and diatom species such as *Rhizosolenia* and *Chaetoceros* which also showed spring peaks in abundance. Plankton abundances based on the microscopy showed more pronounced temporal autocorrelation patterns than eDNA metabarcoding (see OTU taxa in Figure 6).

At genus level, abundant taxa that were identified by microscopy but not as OTUs and vice versa were also observed. An

TABLE 1 | Plankton taxa positively associated with the incidence of fish PGD and mortality. Results are shown when no lag was assumed between fish health and plankton abundances and for lagging the PGD and mortality data by 2, 5, and 10 days behind the environment and plankton species data. Across indicates a significant positive association of the species with either PGD or mortality after accounting for the effect of temperature and oxygen saturation. A gray highlight shows that the effect depended on the site/exposure level. The OTU label indicates where the taxon was detected via molecular means.

	PGD				Mortality			
Taxon	**0 days**	**2 days**	**5 days**	**10 days**	**0 days**	**2 days**	**5 days**	**10 days**
Unicellular eukaryotes								
Attheya OTU						x	x	x
Ceratium	x	x		x	x	x	x	x
Chaetoceros sp.	x	x						
Cylindrotheca OTU			x	x				
Dictyocha speculum				x				
Hematodinium OTU			x					x
Leucocryptos OTU		x			x		x	
Ostreococcus OTU	x	x	x	x				
Prorocentrum micans					x	x		x
Pseudo-nitzschia							x	x
Frustulia OTU							x	
Rhizosolenia setigera	x	x			x	x	x	
Zooplankton								
Bivalvia larvae					x	x		x
Copepods	x	x	x	x	x	x	x	x
Doliolid	x	x	x	x	x	x	x	
Gastropoda	x	x			x	x	x	x
Lizzia blondina				x	x			
Lizzia blondina OTU	x	x						
Oikopleura dioica	x	x	x	x	x	x	x	x
Ophiura larvae	x	x			x	x	x	x
Rathkea octopunctata						x	x	
Sea urchin larvae	x	x						
Vannella OTU	x	x					x	

example of this was the zooplankton species *O. dioica*, where no corresponding OTU was observed from the molecular data. On the other hand, eDNA metabarcoding identified the diatom species *Cylindrotheca* as well as several amoebozoan genera which were not detected in the plankton samples via microscopy. For the species that were identified by both methods, not all genera showed significant correlations between abundances (microscopy) and reads (metabarcoding) (Figure 7).

To further assess congruence between metabarcoding and microscopy in our dataset, we evaluated the relative sensitivity of eDNA and microscopic data for taxa that were clearly identified by both methods on any given day (Table 2). We noted a high level of discrepancy in detection sensitivity (a taxon was identified by one technique, but not the other). For example, over 412 sampling days, spanning both locations, at best *Skeletonema* detection discrepancies were detected in 42% of the samples and worst *Ditylum* in 82% of the samples (Table 2). For those taxa identified by both techniques, the relative sensitivity of each was evaluated as the ratio of respective detection days, which revealed that for some taxa, such as the diatom *Skeletonema*, eDNA was more sensitive while for others, such as the hydrozoan *Lizzia*, microscopy performed better.

4 | Discussion

Our study represents a thorough assessment of the planktonic threats faced by an open-water marine aquaculture species, both in terms of breadth (the number of planktonic taxa

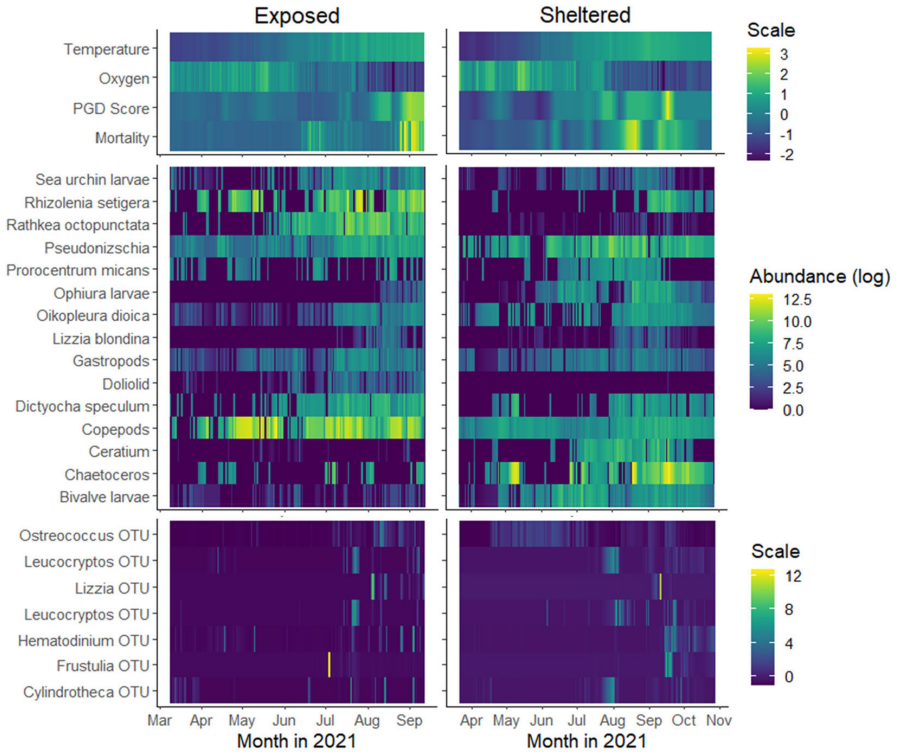

FIGURE 6 | Heatmaps showing the dynamics of variables related to the environment, fish health, and plankton-borne vectors. Plankton was identified either morphologically or molecularly (OTU suffix), and those shown are good predictors of PGD and mortality in at least one of the two sites (exposed and sheltered). All variables have been interpolated, and genus OTUs, fish condition, and abiotic variables have been normalized by zero-centering to enable direct comparisons with plankton data. Abundance was measured in cells L^{-1} or individuals m^{-3} depending on whether the organism was a planktonic unicellular eukaryote (protist) or zooplankton.

identified) and depth (number and temporal frequency of sampling days). We noted a general trend in fish health for the two sites we monitored, with mortality and PGD scores worsening in late summer months (July–September). High sampling frequency and two sites with contrasting environmental characteristics enabled some statistical deconvolution of planktonic species associated with poor fish health from collinear variables such as sea surface temperature and dissolved oxygen. Biological correlates with PGD and mortality were largely site-specific. As such, only the dinoflagellate genus *Ceratium*, copepod larvae, and the hydromedusa *L. blondina* were linked with poor fish health at both sites. Meanwhile, doliolids (classified as *Doliolum nationalis* and *Dolioletta gegenbauri*) were strongly associated with poor fish health at the exposed site, and the appendicularian *O. dioica* was a principal biological correlate with mortality at the sheltered site. Interestingly, the correlation between some planktonic taxa (e.g., *Pseudonitzschia*, *Cylindrotheca*) and poor fish health was noted only when fish health variables lagged behind planktonic dynamics, indicating delayed effects of certain planktonic species on fish condition. Finally, we evaluated the correspondence between molecular (eDNA metabarcoding) and morphological (microscopy) approaches in plankton detection. In general, the approaches were poorly correlated, with eDNA relative read abundances being a poor predictor of microscopic cell/organismal abundance. Relative detection sensitivities were also highly variable, with some important planktonic predictors of fish health being detected by only one of the two techniques deployed (microscopy vs. eDNA).

Temperature and dissolved oxygen have been reported as direct drivers of salmon gill health (Ghosh et al. 2022; Herrero et al. 2022; Remen et al. 2016) and our study confirms this. However, having accounted for the effect of these key abiotic drivers, our analyses across both sites identified numerous biological correlates with poor fish gill health and mortality. Cnidarian gelatinous zooplankton, especially hydrozoans, have been widely reported as drivers of gill insult and salmon mortality (Boerlage et al. 2020). Our morphological analyses detected multiple hydrozoan species—*R. octopunctata*, *L. blondina* (Figure 6), *Obelia* spp., *Ectopleura* sp., *Bougainvillia* sp., *Phialella quadrata*, *Clytia hemisphaerica*, and others (Appendix). The hydroid of *Ectopleura* cf. *larynx*, a cnidarian fouling species with the potential to cause salmon health issues (Bloecher et al. 2018), was identified from our zooplankton samples, presumably released into the water during regular in situ net cleaning in the farms. Only *Rathkea*, *Lizzia*, and *Obelia* were detected in appreciable abundance, and only *L. blondina* correlated with both gill damage (PGD, Exposed Site) and mortality (Sheltered site). *Rathkea* also had some impact on mortality, but the impact lagged by several days after detection of the species. *L. blondina* has been correlated with gill damage in Scotland previously, as have *Obelia* spp. (Kintner and Brierley 2019). Despite the high abundance of *Obelia* across both sites, much higher than *L. blondina* (Appendix), we did not identify any correlation with fish health. Other hydrozoan species such as the siphonophore *Muggiaea atlantica* are known to represent particular threats to salmon gills (e.g., Baxter et al. 2011) and have also been detected in our water samples by eDNA metabarcoding analyses (748

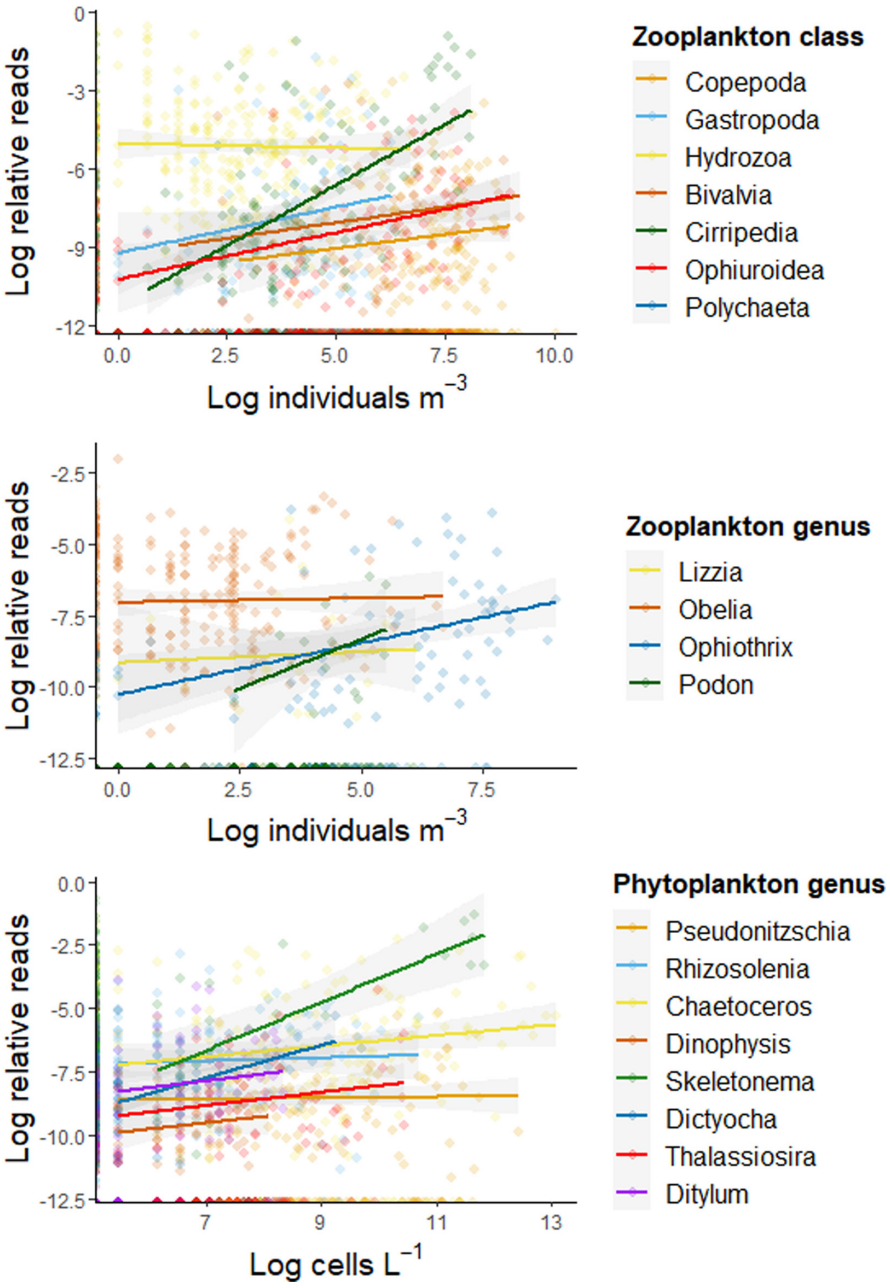

FIGURE 7 | Relationship between abundances from eDNA (log relative reads) and microscopy (either cells L^{-1} for phytoplankton or individuals m^{-3} for zooplankton) for classes and genera that were detected by both methods. Site-specific relationships between eDNA and microscopy abundances for each of these taxa are presented in the Figures S1 & S2 and in Table 2.

reads), while we could not detect their presence via microscopy. The pathophysiology of the different salmon-hydrozoan species-specific interactions might be determined by the mechanical damage of the nematocysts as well as the toxins released (see Bloecher et al. 2018); however, other factors such as jellyfish swarm size, exposure time (Clinton et al. 2021), toxins type, and fish immune response could also have an effect. Moreover, our results show that gelatinous zooplankton (GZP) causing salmon gill disease include not only cnidarians but other groups such as appendicularians and doliolids—which do not have nematocysts to penetrate the gill tissue—suggest that jellyfish envenomation might not be the only trigger of CGD, and certainly calls for further investigation on the mechanisms that determine the pathogenicity of this group.

Diatom blooms have long been understood as drivers of salmon mortality at sea (Albright, Yang, and Johnson 1993; Bell 1943). Here, multiple diatoms (e.g., *Rhizosolenia*, *Chaetoceros*, *Pseudo-nitzschia*) were correlated with PGD and/or mortality. Proposed mechanisms include both mechanical damage of gill tissue by the siliceous spines of diatoms (Albright, Yang, and Johnson 1993) as well as direct toxin production by microalgae species (e.g., Bates et al. 2018). The dynamics of dinoflagellates *Ceratium* and *Prorocentrum* were also negatively associated with fish health. Bloom concentrations of *Ceratium* and *Prorocentrum* have been linked with dissolved oxygen depletion and subsequent fish kills in the past (Azanza et al. 2005; Glibert et al. 2002; Malone 1978). In our study, *Ceratium* and *Prorocentrum* reached maximum concentrations of 3.5×10^4

TABLE 2 | Assessment of congruence between morphological and eDNA metabarcoding plankton data across those genera and broader taxonomic groups that were identified by both methods (e.g., Appendicularia was not detected by eDNA and is thus excluded).

Genus	Notes	Microscopy/eDNA discrepancy	Microscopy/eDNA ID success ratio	Slope (r^2)	Site dependency
Pseudo-nitzschia	3 OTU genera, 1 microscopy genus	60%	2.28	0.964 (1.7%)	No
Rhizosolenia		55%	0.12	0.334 (3.4%)	No
Chaetoceros		65%	0.26	0.030 (6.9%)	No
Dinophysis		69%	3.38	31.689*** (8.5%)	Yes
Skeletonema		42%	0.008	44.631*** (10.2%)	No
Dictyocha		65%	0.43	85.492*** (20.8%)	Yes
Thalassiosira		72%	5	50.670*** (11.3%)	No
Ditylum	*D. brightwellii* by both methods	81%	0.36	0.003 (2.2%)	No
Lizzia	*L. blondina* by both methods	82%	8.12	3.306 (1.4%)	No
Obelia		66%	0.24	7.721* (2.4%)	No
Ophiothrix		69%	4.72	0.081 (7.9%)	No
Podon		68%	129	64.648*** (13.8%)	Yes◊
Copepoda	12 OTU genera, 14 microscopy genera	56%	7.52	0.310 (0.7%)	Yes
Gastropoda	28 OTU genera	57%	7.90	0.292 (1.7%)	No
Hydrozoa	16 OTU genera	77%	0.17	0.308 (10.0%)	No
Bivalvia	20 OTU genera, 2 microscopy genera	61%	3.38	0.073 (1.0%)	No
Cirripedia	4 OTU genera	66%	1.91	11.412*** (19.6%)	Yes
Polychaete	32 OTU genera, 4 microscopy genera	55%	5.57	0.406 (0.3%)	No
Malacostraca	18 OTU genera, 6 microscopy genera	69%	2	0.028 (0.5%)	No

Note: The degree of discrepancy between the two methods is expressed as the percentage of total samples (412) that a genus was identified by only one of the two methods. The ratio of samples identified by microscopy over those identified by eDNA shows the relative strength of each method (>1 indicates that microscopy is more effective whereas <1 indicates eDNA is more effective in identifying this species). The strength of the relationship between eDNA-relative read data and abundance microscopy data is assessed using linear regression that assumes dependency on the exposure level/site ($Taxon_{OTU} \sim Taxon_{microscopy} \times$ Exposure level). The strength of the relationship is expressed by the F-ratio, the significance level (*** significance at 99.9% level, ** at 99%, * at 95% and no asterisk indicates no significance), and the r-squared. ◊ indicates that the taxon was only present on the exposed site.

and 7×10^3 cells/L, respectively, therefore, although high, neither are considered bloom concentrations. However, *Ceratium* consisted of species such as *C. lineatum*, *C. furca*, *C. fusus*, and *C. tripos*, the latter three being quite voluminous dinoflagellates as with their needles or u-shaped with multiple long horns they can reach up to 230 μm in size. Lower concentrations of large *Ceratium* species have been associated to fish kills in the past via mechanical damage to fish gills and secondary infection (Orellana-Cepeda, Granados-Machuca, and Serrano-Esquer 2002) which might be also the case in our study. Finally, the silicoflagellate, *Dictyocha speculum* also had a lagged effect on the incidence of PGD in our study, in agreement with a previous report on this microalga causing mass mortality on farmed *S. salar* in a Galician ria (Prego et al. 2023).

Among the most highly ranked taxa in terms of their correlation with both PGD and salmon mortality were several GZP species, as well as unicellular eukaryotes, never before suggested as harmful to fish. These species provide a first glimpse below the tip of the iceberg in terms of the hidden diversity of planktonic threats to gill health. Doliolids are planktonic filter-feeding tunicates 1–2 mm in length, which appeared strongly associated with mortality and PGD at the exposed site, more so than temperature and DO (Figure 5). Doliolid blooms have been associated with climate-change-related heatwaves (Pinchuk, Batten, and Strasburger 2021), and these numbers broadly tracked temperature in our study. Doliolids are not known to be toxic, although their selectivity for diatoms and ciliates (Frischer et al. 2021) suggests that toxins accumulation might be

occurring in doliolids bodies; this; however, has not been tested to date, to the best of our knowledge. Alternatively, these small GZP could simply adhere to and clog the gill lamellae, obstructing gaseous exchange. Another pelagic tunicate strongly associated with gill disease and mortality, this time at the sheltered site, was the appendicularian *O. dioica*. Again, the role of *O. dioica* as an irritant is far from clear. This species filter feeds on microplankton, including marine viruses, via an extruded cellulose net (Lawrence et al. 2018). *O. dioica* may potentially bioaccumulate heavy metals, or even toxins of algal origin; however, they too can be highly sensitive to such compounds (Calatayud et al. 2018; Torres-Águila et al. 2018). As with doliolids, a mechanism of *O. dioica* toxicity beyond direct obstruction of the gas exchange surface has not been defined yet.

As well as doliolids and appendicularians, several other components of the zooplankton community such as sea urchin larvae, copepods, and gastropods have been identified as potential poor gill health and mortality drivers. As this study initially targeted planktonic groups causing diminished salmon health based on literature (i.e., cnidarians and harmful phytoplankton blooms), morphological identification efforts did not focus at lower taxonomic levels on these common and highly diverse taxa. At the other end of the scale, the amoebozoan *Vanella* and the protist *Leucocryptos* were also incriminated. The correlations we detected, however, suggest that zooplankton-gill health interactions can involve more players and levels of complexity than traditionally expected. A mechanism that might link each class of organism to gill damage is beyond the reach of this study but is clearly an important avenue for further research. However, it is worth noting that the most important correlates with poor gill health and mortality were not limited to hydrozoans or harmful algal species, despite the fact that these groups are the only ones reported in the literature (e.g., Boerlage et al. 2020), and the species most closely monitored by the aquaculture industry (Bickerdike, pers. comm). Indeed, the most important correlates in this study have never before been reported as important drivers of gill health or mortality of farmed Atlantic salmon.

The presence of a planktonic irritant on a given day may not result in an immediate impact on either gill integrity or fish health. Furthermore, gill damage is likely to be cumulative (Boerlage et al. 2020; Østevik et al. 2022) with the condition worsening over the course of spring/summer months, increasing the sensitivity of fish to mortality through physiological stress associated with feeding, medicine treatments, and other handling events. Cumulative damage may be best reflected through PGD score, as AGD scores do seem to improve after freshwater treatments for *N. perurans* (e.g., Parsons et al. 2001). The ability of AGD gill lesions to resolve post-treatment is clearly evidenced in our data, whereby high AGD gill scores, which trigger treatment events, have a strong negative relationship with mortality independently of site ($F = 91$, $p < 0.001$). In contrast, PGD score was positively associated with mortality independently of site ($F = 615$, $p < 0.001$). To capture some of the delayed and/or cumulative impacts of different irritants on gill health, we lagged mortality and PGD behind our planktonic data and assessed how strongly different taxa were associated with poor health outcomes. Several taxa identified in our unlagged ranking analysis also appeared to have significant lagged associations with mortality. However, several previously unrecorded phytoplankton taxa may have a delayed, but nevertheless important, impact on fish health, for example, the diatoms *Cylindrotheca* and *Frustrulia*, the Ochrophyte *Dictyocha*, and the dinoflagellate *Hematodinium*. *Hematodinium* is of particular interest as it is a parasite of a decapod crustacean of economic importance in Scotland and globally (Beevers et al. 2012), although to our knowledge it has never been isolated from Atlantic salmon.

Establishing the value of eDNA in predicting organismal biomass is a long-standing goal of molecular ecological research in freshwater and marine environments (e.g., Bourque et al. 2022; Lamb et al. 2019; Rourke et al. 2022). Experimental work has demonstrated that eDNA concentrations tracked via qPCR (e.g., Bourque et al. 2022) and metabarcoding (Peters et al. 2018) can predict absolute and/or fold changes in plankton biomass. Some success was also reported in the marine environment in field conditions (Ershova et al. 2021; Santi et al. 2021). On the other hand, studies have also highlighted the challenges of amplification biases in multispecies samples leading to noisy compositional metabarcoding data that are unreliable for inference purposes and correlations with abundances from microscopy data (Gold et al. 2023; Kelly, Shelton, and Gallego 2019; Shelton et al. 2023). In our study, results were mixed. For zooplankton taxa, the predictive value of eDNA on biomass was generally poor with the exception of Cirripedia which showed the strongest relationship between microscopy and metabarcoding data with 20% of the variation explained by the linear relationship. There is a stronger relationship between molecular and microscopic estimates of abundance for phytoplankton, especially for more frequently occurring taxa (e.g., *Skeletonema*). The higher biomass disparity within and among zooplankton taxa might have contributed to this result, and experimental validation of the relationship between eDNA detection efficiency and biomass for individual zooplankton species may complement our work. The sensitivity of eDNA and microscopic approaches also varied, with important taxa often clearly identified by one technique and largely missed by another. Examples of this were the diatom *Attheya* that was identified by metabarcoding and which can be confused with the genus *Chaetoceros* during microscopy analysis. Other examples identified by metabarcoding but were not seen in the microscope samples were the diatom *Cylindrotheca*—which was potentially due to long sample preservation times—and the siphonophore *M. atlantica*.

For studies that compare traditional and DNA-based approaches, the conclusion that best serves the end goal of surveying biodiversity is often "use both" (e.g., Santi et al. 2021). In some respects, our study is no exception, and no single approach has a clear advantage. However, several potential improvements, especially to the molecular methodologies, could be considered to improve the detection of species of interest. First, despite mining our amplicon data for doliolid and appendicularian species, it appears that the mlCOIintF-XT/jgHCO2198 primer pair does not amplify tunicate COI with appreciable efficiency. Furthermore, reference sequences in the Universal-databank for Fisheries and Aquaculture cytochrome oxidase I database (https://github.com/uit-metabarcoding/DUFA) for these groups are missing or is incomplete,

and further curation may be required (Præbel and Cañestro., pers. comm). Second, although the aim of this study was to survey planktonic diversity by as unbiased a means as possible, it is clear that a number of key eukaryotic salmon pathogens are missing from the molecular data, and as such fish farm managers should interpret eDNA metabarcoding data alone with caution. AGD gill scores and gill qPCR data indicate that *N. perurans* is abundant at both sites (see Data S1), and, as in previous work (Bridle et al. 2010), *N. perurans* should be readily detected from the water column. Similarly, sea louse counts on salmon (*L. salmonis* / *Caligus elongatus*) indicate the presence of these parasites at both sites. DNA from adult and juvenile lice should also be abundant in the water column (e.g., Krolicka et al. 2022). However, unambiguous *N. perurans* reads were extremely rare in the dataset (19 total, see Appendix), as were those for *L. salmonis* (976 total) and *C. elongatus* (18 total). For context, we found 876 red deer (*Cervus elaphus*) reads in the data, presumably washed in from streams and rivers. Clearly "non-target" species abound in the data. Billions of reads in our dataset, for example, were assigned to free living, apparently non-pathogenic amoeba (e.g., *Cunea*, *Parvamoeba*), and the read depths required to per sample to "sequence through" this biological noise using universal metabarcoding markers are impractical. As such, monitoring of specific planktonic threats by molecular means may be better achieved by targeted—for example, qPCR (Bridle et al. 2010; Krolicka et al. 2022)—and semi-targeted—for example, clade-specific—metabarcoding approaches (Dario et al. 2017). Target and non-target species ID could also be approached by using multiplexing primers of 18S, cytochrome, and other housekeeping markers. However, it is clear from our data that the discovery phase in this area is far from over, and metabarcoding still has a role.

In this study, we sampled two aquaculture sites intensively over a single growing season using microscopy and metabarcoding. We aimed to achieve unbiased planktonic sampling and then applied rigorous statistical models to link different planktonic taxa to salmon health outcomes. Crucially, respective planktonic exposure profiles were both divergent and idiosyncratic, as were the apparent biological correlates of poor gill health. Aquaculture site characteristics, sheltered or exposed, may account for some of the differences observed. However, to make generalizations about the extent of the biological threats to salmonid aquaculture further unbiased studies at multiple sites across multiple production cycles are required. Improving the resolution of the eDNA metabarcoding approach is critical considering the time and costs associated with this analysis were about a third to those associated with the microscopy analysis of plankton samples since the latter required two different experts to analyze the zooplankton and phytoplankton samples. In parallel, to better understand the mechanisms of gill damage and mortality and which organisms drive them, a more detailed monitoring of salmon gill health is required (e.g., gene expression, histopathology) to help disentangle from the role of direct microalgae toxicity and help set abundance threshold for mitigation purposes. Indeed, new understandings will lead to new approaches for mitigation. These could include protection barriers, aeration technology, early warning signals, new medicinal interventions, and, eventually, methods to enhance fish and gill resilience through functional feeds or selective breeding programs that will enable salmonid aquaculture to thrive in a continuously changing climate.

Author Contributions

Sofie Spatharis, María Algueró-Muñiz, Kim Præbel, and Martin Llewellyn made major contributions to the conception or design of the study; Sofie Spatharis, María Algueró-Muñiz, Toni Dwyer, Michele de Noia, Calum Johnstone, Jennifer Welsh, Annabell Macphee, Marta Mazurkiewicz, and Clara McGhee collaborated in the acquisition of the data; Martin Llewellyn, Sofie Spatharis, María Algueró-Muñiz, Bachar Cheaib, Yee Wan Liu, Brendan A. Robertson, and Marta Mazurkiewicz collaborated in the analyses of the data; María Algueró-Muñiz, Sofie Spatharis, Ralph Bickerdike, Hervé Migaud, Kim Præbel, and Martin Llewellyn have collaborated in the interpretation of the data. Sofie Spatharis, Martin Llewellyn, and María Algueró-Muñiz led the writing process of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data used and generated during this study is publicly available in https://doi.org/10.5061/dryad.08kprr5bp.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.