Biotic signals associated with benthic impacts of salmon farms from eDNA metabarcoding of sediments

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Abstract

Environmental DNA (eDNA) metabarcoding can rapidly characterize the composition and diversity of benthic communities. As such, it has high potential utility for routine environmental assessments of benthic impacts of marine finfish farming. In this study, 126 sediment grab samples from 42 stations were collected along an organic enrichment gradient at six salmon farms in British Columbia, Canada, and benthic biotic community changes were assessed by both eDNA metabarcoding of metazoans and macrofaunal polychaete surveys. The latter was done by analyzing 11,466 individuals using a combination of morpho-taxonomy and DNA barcoding. Study objectives were to: (1) compare biotic signals associated with benthic impacts of salmon farming in the two data types; and (2) identify potential eDNA indicators to facilitate eDNA-based monitoring in Canada. Across both data types, alpha diversity parameters were reduced in sediments near fish cage edge and were negatively correlated with pore-water sulphide concentration. Presence/absence of known indicator taxon Capitella generally agreed well between the two methods despite that they differed in both the volume of sediment sampled and the molecular marker used. In eDNA data, there was a strong negative correlation between Nematoda OTU richness and pore-water sulphide concentration, and multiple approaches were used to identify OTUs related to organic enrichment statuses. We demonstrate that eDNA metabarcoding generates biotic signals that could be leveraged for environmental assessment of benthic impacts of fish farms in multiple ways: both alpha diversity and Nematoda OTU richness could be used to assess the spatial extent of impact, and OTUs related to organic enrichment could be used to develop a local biotic index.

Introduction

Benthic macrofauna are abundant in marine and freshwater ecosystems and play vital roles in nutrient cycling and secondary production (Snelgrove, 1997, 1998). For example, macrofauna decompose organic inputs by feeding directly on organic matter, and they promote metabolism of pollutants by bacteria through biological transport and by increasing the oxygen supply in sediments (Pant, Negi, & Kumar, 2017; Sanz-Lázaro & Marín, 2011; Snelgrove, 1998). Because macrofauna are important to ecosystem processes, can be identified by microscopy, and have differing tolerances to enrichment gradients, they have been used widely to assess environmental impacts associated with human activities, including marine finfish farming (Keeley, Forrest, Crawford, & Macleod, 2012; Sanz-Lázaro & Marín, 2011), oil-drilling (Denoyelle, Jorissen, Martin, Galgani, & Mine, 2010), and mining (Josefson, Hansen, Asmund, & Johansen, 2008). Macrofauna assessments have also been used to monitor environmental recovery from these activities (Daan, Booij, Mulder, & Weerlee, 1996; Machado et al., 2018; Zhulay, Reiss, & Reiss, 2015).

To date, characterization of macrofauna communities has been done by traditional morpho-taxonomic identification, which is very time- and labour-consuming and relies heavily on highly specialized taxonomists.

These factors intrinsically limit the speed and magnitude of sample throughput that can be achieved by traditional taxonomic approaches (Bourlat et al., 2013; Danovaro et al., 2016; Goodwin et al., 2017; Ji et al., 2013; Pawlowski et al., 2018). Furthermore, individuals that are damaged during mechanical collection and sieving processes as well as with poorly described life stages may be misidentified by visual identification (Danovaro et al., 2016; Goodwin et al., 2017). Exacerbating these challenges is that species that are difficult to distinguish by morpho-taxonomy are commonly present in these communities (Knowlton, 1993), and if improperly identified could lead to underestimation of biodiversity and/or misleading interpretations about indicator species used for environmental assessment (Dean, 2008; Nygren, 2014).

DNA barcoding alleviates the reliance on morphological characters for taxonomic identification and can offer several benefits including higher taxonomic resolution of closely-related or cryptic species and the ability to verify morphologically-identified taxa (Nygren, 2014; Westheide & Schmidt, 2003; Witt, Threloff, & Hebert, 2006). It can also be used to characterize the taxonomic composition of opportunistic indicator complexes (Silva et al., 2017). However, it is still relatively time- and cost-intensive due to the one-by-one nature of the workflow: samples need to be manually sorted into individual specimens and the cost of Sanger DNA sequencing.

Environmental DNA (eDNA) metabarcoding is a high-throughput, rapid, cost-effective, and relatively new method of characterizing species composition in complex environmental samples (Shokralla, Spall, Gibson, & Hajibabaei, 2012; Taberlet, Bonin, Zinger, & Coissac, 2018; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). It has been proven to be useful for detecting invasive (Klymus, Marshall, & Stepien, 2017) and rare species (Pikitch, 2018), assessing biodiversity for conservation (Lacoursière-Roussel et al., 2018; Stat et al., 2019), and monitoring environmental health status (Laroche et al., 2016; Pawlowski, Esling, Lejzerowicz, Cedhagen, & Wilding, 2014). Environmental DNA metabarcoding has been used to characterize benthic macrofauna responses to human activities associated with marine finfish aquaculture (Lejzerowicz et al., 2015), offshore oil-drilling (Lanzen, Lekang, Jonassen, Thompson, & Troedsson, 2016), oil spills (Xie et al., 2018), and chemical contamination (Xie et al., 2017). It has the potential to supplement traditional morpho-taxonomic analysis of macrofauna from sediments for environmental assessment and biomonitoring (Aylagas, Borja, & Rodriguez-Ezpeleta, 2014; Pawlowski et al., 2018), and to increase the spatial and temporal scales at which biomonitoring can realistically be done (Gibson et al., 2015). Available studies comparing eDNA metabarcoding with traditional morpho-taxonomy have only used visual morpho-taxonomy of macrofaunal individuals without the support of DNA barcoding for resolving morphologically similar (or cryptic) species. This makes it difficult to compare macrofauna inventories among studies, particularly for taxonomic groups prone to cryptic speciation including Capitellidae, a family that is especially relevant to benthic biomonitoring. In the absence of an objective means of classifying species based on morphology, there will inevitably be variation among studies as to how taxa were classified when morpho-taxonomy alone is used.

Benthic organic enrichment is a significant environmental issue associated with marine finfish aquaculture (Forrest et al., 2007; Wilson, Magill, & Black, 2009). It can influence faunal diversity and abundance, causing population shifts towards opportunistic taxa (Pearson & Rosenberg, 1978) thereby eliminating a diverse community and the functions originally provided by it within the ecosystem (Snelgrove, 1997, 1998). As such, benthic impacts of finfish farming are subject to regulatory control and routine monitoring in many countries (Wilson et al., 2009). In New Zealand, Norway, and Scotland, biological indices based on morpho-taxonomic macrofauna data are implemented for regulatory purposes (Keeley et al., 2012; Maurer, Nguyen, Robertson, & Gerlinger, 1999). To date, a regulatory biological index for evaluating benthic impacts has not been implemented in Canada. Geochemical indicators (e.g. sediment pore-water sulphide concentration) are used for routine regulatory benthic soft sediment monitoring (Hargrave, 2010; Hargrave, Holmer, & Newcombe, 2008), and macrofauna are analyzed only under certain benthic conditions (AAR, 2016; Wildish, Pohle, Hargrave, Sutherland, & Anderson, 2005). The addition of a biotic measure to routine assessments of soft sediments to complement information obtained by geochemistry would advance the state of the science of benthic monitoring in Canada, providing regulators additional information for decision-making. This could come in the form of eDNA-based data, either by metabarcoding or assays targeting particular taxa

once eDNA indicators are identified.

Here we advance existing knowledge on the responses of benthic metazoans to organic enrichment with the goal of testing the potential application of eDNA metabarcoding to benthic impact assessments of fish farms. At seven stations along a distance gradient at each of six salmon farms in British Columbia, Canada, we collected sediment samples for eDNA metabarcoding analysis of metazoans, geochemistry measurements, and morpho-taxonomy combined with DNA barcoding analyses. For morpho-taxonomy and DNA barcoding, we focused on Polychaeta as this was the most abundant group of species observed and taxa therein are known to be good indicators of benthic impacts (Dean, 2008; Tomassetti & Porrello, 2005), especially the *Capitella capitata*complex (Dean, 2008; Wildish et al., 2005). The specific objectives of this study were to: (1) compare biotic signals associated with benthic impacts of salmon farming between morpho-taxonomic polychaete and eDNA metabarcoding data; and (2) identify potential eDNA indicators that could form the basis for development of eDNA-based monitoring methods in Canada. We analyzed biotic signals associated with benthic impacts of fish farms in eDNA metabarcoding data and macrofaunal polychaetes data, and used multiple methods to analyze the relationship between operational taxonomic units (OTUs) and organic enrichment.

Materials and Methods

Sediment collection

Sediment samples were collected in 2016 at six marine finfish farms located in BC, Canada: Baxter Islet (BI, May 10), Bedwell Sound (BS, April 13), Midsummer Island (MI, June 8), Mussel Rock (MR, April 12), Plover Point (PP, May 11), and Venture Point (VP, June 14). Samples were collected at seven stations along a transect in a dominant current direction initiated from the fish cage edge. The seven stations were intended to be 0 m, 15 m, 30 m, 60 m, 90 m, 125 m, and 500 m from cage edge. Samples were collected from those distances from cage edge where possible; the exception was the reference station at MI, which was 2260 m from cage edge as it was the closest reference station found with soft (and thus 'grab-able') sediments. Detailed site information was provided in Table S1.

A total of three sediment grab samples were collected at each station using a Van Veen Grab Sampler (0.1 m^2, 24L) (Blomqvist, 1991; Gage & Bett, 2005). Pore-water sulphide concentration and redox potential for each grab were measured prior to the collection of other environmental variables to avoid oxygen injection into the sediment. Sediments were classified into four different sulphide concentration categories according to the Canadian Aquaculture Activities Regulations (AAR), as follows: anoxic ($>$ 4,500 µM), hypoxic (1,300 – 4,500 µM), oxic B (700 – 1,300 µM), and oxic A ($<$ 700 µM) (AAR, 2016). How each station was sampled is presented schematically in Figure S1 and summarized here. One grab sample was devoted entirely to macrofauna analysis (details below); each of the other two grabs were used to collect samples for geochemical analyses (porosity and organic content) and three eDNA samples, which were collected from the top 2 cm of sediment. The samples for geochemical analyses were collected using spatulas and eDNA samples were collected using modified 60 cc syringe-cores according to the sediment and meiofaunal methods detailed in Sutherland *et al* . (2007). Pore-water sulphide concentration, redox potential, porosity and organic content were measured following protocols used in Sutherland *et al* . (2007). To avoid cross-contamination during sample collection, the grab sampler was rinsed in salt water and visually inspected for cleanliness between grabs, samples were collected from the center of the grab only, and syringes were cleaned using diluted bleach solution (final concentration of 3% sodium hypochlorite) between farms. In summary, total numbers of the various sample types collected from a total of 42 stations were: 42 benthic macrofauna samples; 84 samples measured for porosity and organic content; 126 samples analyzed for sediment pore-water sulphide concentration and redox potential; and 252 samples analyzed for eDNA metabarcoding.

Collection and morphological sorting of macrofauna

The macrofauna grab collected at each station was transferred to a sealed plastic bin and transported to an onshore sieving station. A portion of each sediment sample was transferred to a 1 mm mesh sieve table and washed gently with running water. Once the sediment was washed through the sieve, macrofauna were

gently removed with tweezers and transferred to a 500 ml container containing 95% ethanol. This process was carried out until the entire grab sample from each station was sieved. In cases where excess debris (e.g. 'shellhash' or rocks) remained on the sieve that might influence the search for macrofauna, this debris was collected into containers with ethanol for further sorting in the lab.

Macrofauna specimens from each grab were sorted into morphospecies. We only analyzed vermiform macrofauna (e.g. Polychaeta), which comprised the majority of total macrofauna abundance across the aquaculture sites. All individuals of each morphospecies from each grab were separated for counting and downstream DNA barcoding. This was done by one person to maximize consistency. Morphospecies were photographed and defined when first encountered to allow recognition across all farms and grabs. Morphospecies were defined with the aid of taxonomic references (Banse & Hobson, 1974; Dutch et al., 2014; Hobson & Banse, 1981) and/or were identified by Leslie Harris from the Los Angeles County Museum via specimen images uploaded to the iNaturalist platform (*https://www.inaturalist.org/*). After initial sorting of all stations, each morphospecies was re-examined across all farms and stations to confirm consistency of morphospecies assignment. Generally, one or two specimens from each morphospecies at each station were picked for DNA barcoding; however, in cases where morphospecies delineation was less clear, additional specimens were picked for genetic confirmation.

DNA barcoding analysis of macrofauna

A total of 726 individuals were photographed. Primers amplifying the*cytochrome c oxidase I* (*COI*) gene were used for DNA barcoding as this is the standard DNA barcoding marker (Hebert, Cywinska, Ball, & deWaard, 2003); it performs very well for species level discrimination and has a well-populated reference database (the Barcode of Life Data (BOLD) System) (Ratnasingham & Hebert, 2007). A piece of the tail-end was removed for DNA extraction, and the remaining sample was placed in a 1.5 mL tube or larger container as needed. DNA was extracted using DNeasy 96 Blood & Tissue Kits (Qiagen). The COI C_VF1LFt1 and C_VR1LRt1 primers were tried first (Ivanova, Zemlak, Hanner, & Hebert, 2007); samples that failed to amplify or sequence with these primers were amplified with polyLCO and polyHCO primers (Carr, Hardy, Brown, Macdonald, & Hebert, 2011).

To ensure polychaete taxonomic data generated here can be compared to other studies across spatial scales and over time, which is difficult with complex taxonomies, the BOLD Barcode Index Number (BIN) system was adopted to classify barcoded polychaetes (Ratnasingham & Hebert, 2013). When a barcode was too short for automatic inclusion into a BIN by BOLD, it was manually applied to the appropriate BIN via the identification portal of BOLD based on sequence similarity. There were sixteen instances where one morphospecies produced sequences that fell into multiple BINs. When this occurred, the sequenced specimens were examined and then all individuals for that morphospecies were re-examined to determine if they could be appropriately assigned to the identified BINs. There were six occasions where there were 2-5 genetic groups revealed by DNA barcoding that were not visually distinguishable upon re-inspection of specimens, such that it was not possible to assign them confidently into a BIN. In these cases, all specimens were 'lumped' into what we refer to as a 'combined BIN' representing that morphospecies. In a few instances there were individual specimens for which no sequence was obtained but for which a taxonomic name could be confidently assigned using traditional morpho-taxonomic methods. Two alpha diversity parameters describing species richness, Shannon and Simpson indices, were calculated for the macrofauna data using the*vegan* R package (Oksanen et al., 2017).

eDNA metabarcoding

Environmental DNA was extracted from approximate 0.25 g of sediment using the DNeasy PowerSoil Kit (QIAGEN), with one DNA extraction control for every 11 sediment samples. For metabarcoding, the V1 and V2 regions of the *18S rRNA* gene were amplified using primers (F04mod: GCTTGWCTCAAAGATTAAGCC; R22mod: CCTGCTGCCTTCCTTDGA) (Blaxter et al., 1998). We used this 18S rRNA marker rather than a COI marker for eDNA metabarcoding because: (1) this marker has been used successfully to characterize benthic metazoans (e.g. Fonseca et al., 2010; Sinniger et al., 2016); and (2) it amplifies eu-

karyotes only, whereas amplicon sequencing targeting eukaryotes using COI markers typically returns a high percentage of off-target bacterial reads due to non-specific amplification (Collins et al., 2019; Weigand & Macher, 2018). To multiplex, each synthesized primer starts with two or three Ns, followed by an eight-base tag and the gene-specific primer sequences (He, Sutherland, Pawlowski, & Abbott, 2019). PCR protocol and program followed He et al. (submitted). Three PCR replicates were used to amplify each eDNA sample (using the same tagged primers) and replicates were combined after PCR. PCR products from 21 eDNA samples, one DNA extraction control and one PCR negative control were combined into a mixture that was then purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using SYBR Green I (Thermo Fisher Scientific) with a Tecan Infinite M200PRO microplate reader. In total, 12 PCR product mixtures were made. Then, 1 μg of purified PCR mixture was used for library preparation using the TruSeq DNA PCR-Free Library Prep Kit (Illumina). After library preparation and quantification, four libraries were pooled for sequencing using one MiSeq Reagent Kit v3 (600-cycles) (Illumina). In total, three MiSeq Reagent Kit v3 (600-cycles) were used to sequence the 252 sediment samples.

Bioinformatics and statistical analyses

Bioinformatics analyses followed the pipeline used in He *et al* . (2019). Briefly, AdapterRemoval v2 was used to remove adapters in raw sequences (Schubert, Lindgreen, & Orlando, 2016). Sequences were demultiplexed using the*ngsfilter* command of obitools, and sequences for each sample were split using the *obisplit* command (Boyer et al., 2016). Unpaired reads for each sample were removed using modified fastqCombinePairedEnd.py (*https://github.com/enormandeau/Scripts/blob/master/fastqCombinePairedEnd.py*). Paired sequences of each MiSeq run were imported into QIIME 2 separately (Bolyen et al., 2018). Sequence quality control was conducted and the amplicon sequence variant (ASV) tables were constructed using the DADA2 plugin of QIIME 2 (Callahan et al., 2016). Sequences from each MiSeq run were processed separately by DADA2, and the three resulting ASV tables were merged.

De novo OTU clustering based on the ASV count table and sequences was performed at 97% identity using the *vsearch* plugin of QIIME 2 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). The 97% identity was used as it has been shown to be a good cut-off for metazoans from a mock community analysis (Fonseca et al., 2010). Although using ASVs can retain more biological information (Callahan, McMurdie, & Holmes, 2017), we did OTU clustering to: 1) allow a valid comparison between the number of taxa detected using eDNA and morpho-taxonomy; 2) reduce the number of features to make it easier to manually verify if they were derived from benthic metazoan taxa or not; and 3) identify indicator taxa at a higher level than the ASV level. In the raw OTU table, the average number of reads in sediment samples was 168,454 while the average number of reads in controls (DNA extraction and PCR negative controls) was 23. Controls were then filtered out from the OTU table using the *feature-table* plugin. The OTUs with less than 10 reads or present in only one sample were discarded to generate the all-community OTU table (OTU_A). Taxonomy assignment was performed using the *q2-feature-classifier* plugin of QIIME2 and the Silva database v132 (Bokulich, Kaehler, et al., 2018; Quast et al., 2013). Based on taxonomy assignment results (Table S2), non-metazoan OTUs (i.e. not assigned to*D_3__Metazoa (Animalia)*) and two OTUs (one Atlantic salmon OTU and one chicken OTU) that were considered direct farm products were removed from the all-community OTU table to generate the metazoan OTU table (OTU_M). Further, OTUs that were considered non-benthic metazoans (e.g. pelagic) (Table S3) based on their role in oceanographic ecosystems (Sutherland, 1991; Sutherland, Leonard, & Taylor, 1992), including those identified in Lanzén et al. (2016), were removed from the metazoan OTU table to generate the benthic metazoan OTU table (OTU_{BM}). Downstream analyses used the OTU_{BM} table unless stated otherwise.

The feature sequences were used to generate a rooted phylogenetic tree using the align-to-tree-mafft-fasttree plugin of QIIME2. For alpha diversity analysis, rarefaction plotting was performed on the OTU_{BM} table (Figure S2). The OTU_{BM} table was then rarefied to 500 sequences per sample for estimation of two alpha diversity richness parameters: Faith's Phylogenetic Diversity (Faith's PD) (Faith, 1992) and Observed OTUs. Correlations between various biotic parameters (i.e. alpha diversity, Nematoda and Polychaeta OTU richness, relative abundance of *Capitella capitata*) and pore-water sulphide concentrations were tested using

Spearman's correlation analyses as data transformation did not lead to normal distributions.

Linear mixed effect models were used to independently describe the relationships between distance from cage edge and two alpha diversity parameters (Faith's PD and Observed OTUs) using the R package *lme4* (Bates, Mächler, Bolker, & Walker, 2015). Distance was set as a (continuous) fixed effect and farm was stated as a random effect for each model using the *lmer* function call *response_variable ~ distance + (1|farm)* . Generalized linear mixed effect models based on Poisson distribution were used to independently describe the relationships between distance from cage edge and Nematoda OTU richness and Polychaeta OTU richness using the package *lme4* . Distance was set as a (continuous) fixed effect and farm was stated as a random effect for each model using the *glmer* function call *response_variable ~ distance + (1|farm)* . Model fit assessed from QQ-plots of residuals showed the data generally conformed to the assumed distributions for all models, and only the Nematode OTU richness deviated very slightly.

Three separate analyses were performed to identify OTUs associated with benthic impacts of salmon aquaculture. First, a one-way ANOVA was performed to analyze the difference in relative abundance of each OTU in the OTU_{BM} table among organic enrichment statuses using the STAMP software, with P values corrected using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995; Parks, Tyson, Hugenholtz, & Beiko, 2014). Second, the OTU_{BM} table was transformed to a presence/absence table, and the Pearson's phi coefficient of association was calculated to elucidate associations of particular OTUs with sediments of a particular organic enrichment status using the *multipatt* function of the R package *indicspecies* with the *func* set 'r.g', *duleg* set 'TRUE' and *nperm* set '9999' (Cáceres & Legendre, 2009). Again, these P values were corrected using the BH method. Third, supervised machine learning was performed using the *q2-sample-classifier* plugin of QIIME2 (Bokulich, Dillon, et al., 2018), with the *random forest* method and *–p-n-estimators* set to 1000. For machine learning, the OTU_{BM} table and organic enrichment status based on pore-water sulphide concentration for each sample were used to investigate how accurately eDNA data could predict organic enrichment status and the relative importance score of each OTU for this prediction.

Results

Morpho-taxonomy and DNA barcoding of Polychaeta

A total of 11,466 macrofaunal individuals from 42 grabs across six farms comprised 82 Polychaeta and three non-Polychaeta vermiform morphospecies (Table S4). Combining morpho-taxonomy with BIN-based taxonomy derived from DNA barcoding analyses lead to the identification of a total of 150 taxa plus 6 'combined BINs' (described earlier), all of which were Polychaeta except five taxa. A total of 11,445 Polychaeta individuals were counted, with 1279, 431, 3395, 763, 1029, and 4548 collected from BI, BS, MI, MR, PP, and VP farms, respectively (Table 1). The number of Polychaeta collected at each station varied from one (BS, 0 m) to 984 (VP, 30 m). All but two of the Polychaeta taxa sampled could be assigned to at least the family level via barcode analysis, which determined there were 32 Polychaeta families sampled. Numbers of individuals for each Polychaeta family were summarized in Table S5. The most abundant family was Capitellidae, with 7,929 individuals sampled (representing 69% of all sampled polychaetes), 99% of which (N = 7,843) were assigned to the *Capitella* genus. The second most abundant family was Spionidae, which had dramatically fewer individuals (N = 466; 4% of total polychaetes sampled).

Taxonomic composition of eDNA metabarcoding data

There were 42.30 million eukaryote sequences in the OTU_A table, which clustered into 2,239 OTUs. The number of eukaryotic reads per sample varied from 43,498 to 355,828, with an average of 167,872. The most dominant taxon was Ochrophyta, represented by 26.72 million reads and accounting for 63.2% of total reads; the majority of them (26.64 million) were assigned to diatoms (Diatomea). Metazoa was the next most dominant taxon in the OTU_A table, with 12.57 million sequences accounting for 29.7% of total reads across samples. Within the OTU_A table, there were 6,371 reads assigned to Atlantic salmon and 9,959 reads assigned to chicken (a common ingredient in fish food). When chicken reads were detected, they tended to be present at stations close to cage edge, with more reads detected at cage edge (i.e. 0 m) than other stations (Table S6). Atlantic salmon reads had a similar pattern at most farm sites.

Excluding non-metazoan reads and presumed farm contamination reads (i.e. from salmon and chicken), there were 12.55 million metazoan reads in the OTU_M table, which clustered into 400 metazoan OTUs. Metazoan sequences numbered 1.60, 1.51, 1.91, 1.17, 1.65, and 4.71 million at BI, BS, MI, MR, PP and VP farm sites, respectively, which accounted for 20.5%, 26.3%, 30.6%, 17.7%, 16.8%, 77.4% of total reads per farm, respectively (Table1). Most metazoan reads were Calanoida (pelagic copepods), which had 9.11 million reads across samples.

Benthic metazoans were represented by 3.42 million reads clustered into 367 OTUs (Table S7). The number of benthic metazoan sequences was 913,866, 163,707, 646,120, 253,574, 58,500, and 1,388,311 at BI, BS, MI, MR, PP and VP, respectively. In the OTU_{BM} table, Nematoda was the most abundant phylum at BI, BS, MI and MR, accounting for 30.9%-62.5% of reads, while Arthropoda and Annelida were most abundant at PP and VP, respectively (Figure 1). At the order level, the most abundant order of Polychaeta was Capitellida in both DNA barcoding and eDNA metabarcoding data (Table S8).

Alpha diversity

For alpha diversity estimates based on macrofaunal polychaetes data and eDNA benthic metazoan data, distant stations generally had higher alpha diversity than stations close to cage edge for both datasets (Figure 2). Alpha diversity richness parameters were negatively correlated with pore-water sulphide concentration, with coefficients ranging from -0.62 to -0.48 (Figure 3, Figure S3). The LMM analyses showed positive linear relationships between the two alpha diversity parameters generated from eDNA data and distance from cage edge (Figure S4).

Potential indicator taxa: Nematoda and Polychaeta

There were 175 Nematoda OTUs and 38 Polychaeta OTUs in the OTU_{BM} table. Polychaeta are commonly used for morpho-taxonomic analysis for benthic impacts of fish farming; however, interestingly here we found a much stronger correlation between Nematoda and pore-water sulphides than Polychaeta. The correlation between Nematoda OTU richness and pore-water sulphide concentration was -0.86, whereas that between Polychaeta OTU richness and pore-water sulphide concentration was -0.38 (Figure 4). The GLMM analyses showed a positive relationship between Nematoda OTU richness and distance from cage edge, but no relationship was identified between Polychaeta OTU richness and distance (Figure S4). Across all samples, Nematoda OTU richness increased along distance from cage edge. Within each farm, the correlation coefficients between Nematoda OTU richness and distance from cage edge varied from 0.55 to 0.86 (Figure S5).

Capitella polychaetes were found in both morphotaxonomic macrofaunal data and eDNA metabarcoding data; they were present in 29/42 and 25/42 of sampled stations as revealed by macrofaunal and eDNA metabarcoding analyses, respectively (Figure 5). Presence/absence of *Capitella* at each station of BS, MI and VP agreed between macrofaunal and eDNA data. There was one station at MR (90 m) where *Capitella* was present in macrofaunal data but not eDNA data; conversely, there was one station at BI (60 m) where *Capitella* was present in eDNA data but not macrofaunal data. Patterns at PP were markedly different: macrofaunal analyses found *Capitella* at six stations, with a proportion in Polychaeta individuals ranging from 0.38% to 82.5% among stations, whereas *Capitella* were present only at two stations in eDNA data. *Capitella* was not detected at reference stations in either macrofaunal or eDNA datasets. The correlation coefficient between pore-water sulphide concentration and relative abundance of *Capitella* in eDNA data was 0.28 (Figure 4)

OTUs related to organic enrichment status

ANOVA revealed six OTUs with significant differences in relative abundances among organic enrichment statuses (Table 2). Of these, one OTU showed highest relative abundance in oxic A sediments and one showed highest relative abundance in oxic B sediments, and both of these two OTUs were taxonomically assigned to a nematode species *Sabatieria punctata* . The other four OTUs showed higher relative abundances in hypoxic and/or anoxic sediments.

Pearson's phi coefficient analyses revealed 35 OTUs correlated significantly with organic enrichment status, with 26, eight, zero, and one OTUs for oxic A, oxic B, hypoxic, and anoxic categories, respectively (Table 3). Machine learning returned an overall prediction accuracy for organic enrichment status based on pore-water sulphide concentration of 0.77, with an accuracy of 0.91, 0.64, 0.40, and 0.85 for oxic A, oxic B, hypoxic, and anoxic samples, respectively. The three OTUs with highest importance scores for prediction of organic enrichment status were assigned to *Sabatieria punctata* , *Capitella capitata* , and *Gunnarea capensis* , respectively (Table 4); all these three OTUs showed preference for oxic B sediments. The OTU (OTU id: c94fc2ac2f0dc7c32b3decffc60bfc12) with highest importance score for predicting organic enrichment status had highest relative abundance in oxic B sediments.

Discussion

Benthic impacts of marine finfish farms have been studied for decades (e.g. Hargrave, Duplisea, Pfeiffer, & Wildish, 1993). To improve benthic monitoring of fish farm impacts, novel approaches are being developed including new biological indices (Keeley et al., 2012) and more accurate measurements of pore-water sulphide concentration (Cranford, Brager, & Wong, 2017). Recently, eDNA metabarcoding based methods (e.g. including machine learning and using metabarcoding data in biotic indices) for benthic assessment have been proposed (Cordier et al., 2017, 2018; Keeley, Wood, & Pochon, 2018; Lejzerowicz et al., 2015). In this study, we further determined eDNA metabarcoding to be a useful complement to traditional macrofaunal benthic assessments of fish farms by analyzing biotic signals associated with benthic impacts of salmon farming in macrofaunal polychaetes data and eDNA metabarcoding data.

Polychaetes are an abundant taxonomic group in benthic macrofauna (Dean, 2008; Tomassetti & Porrello, 2005) and opportunistic polychaete complex (OPC) are considered good indicator species for environmental monitoring as they respond well to organic enrichment gradients (Pocklington & Wells, 1992). In this study, alpha diversity (richness) calculated using genetically identified polychaete data was consistently reduced near salmon cages compared to further out. This was consistent with previous findings that most polychaetes are sensitive to organic enrichment (Tomassetti & Porrello, 2005), and species richness of benthic fauna was reduced by marine finfish farming (Holmer, Wildish, & Hargrave, 2005; Neofitou, Vafidis, & Klaoudatos, 2010). The presence of *Capitella* at three stations close to cage edge (i.e. 0 m, 15 m, and 30 m from cage edge) and its absence at reference stations at all farm sites further supports that this genus is a good indicator for organic enrichment caused by finfish farming (Pearson & Rosenberg, 1978).

Consistent with results obtained using macrofaunal polychaete data, evidence of benthic biodiversity being impacted by fish farming across the six farms was also apparent using eDNA metabarcoding: there was a negative correlation between alpha diversity parameters and sediment pore-water sulphide concentration, and an increase in alpha diversity at distant stations. This indicates that alpha diversity parameters estimated from eDNA data can be used to analyze the spread (or 'footprint') of benthic impacts at marine finfish farm sites. While other studies have shown eDNA metabarcoding generally provides similar conclusions for environmental assessments to those provided by morpho-taxonomy (Lanzén et al., 2016; Lejzerowicz et al., 2015; Pawlowski et al., 2014, 2016; Pochon et al., 2015), the community composition as revealed by these two methods can be very different (Cowart et al., 2015; Kelly et al., 2017). This was indeed the case in this study, as we detected a much higher number of Polychaeta species using morpho-taxonomy combined with DNA barcoding (145 taxa) than using eDNA metabarcoding (38 OTUs). The major reason causing this could be the difference in amount of sediments used: eDNA metabarcoding was conducted using ca. 1.5 g sediments per station (six 0.25 g samples) while macrofaunal Polychaeta were collected from 24 liters of sediments per station. Thus, it is not overly surprising that more species were detected by picking specimen from sieved sediments, despite that eDNA metabarcoding can detect much smaller species including meiofauna and microfauna. Another important reason could be the primers biases (Zinger et al., 2019): primers used for eDNA metabarcoding may not be very efficient for Polychaeta and/or DNA from other taxonomic groups (e.g. diatoms) were more competitive for PCR amplification. In addition, COI evolves more quickly than 18S and thus is more effective at differentiating species; this also likely contributed to the difference (Tang et al., 2012). Last but not least, public databases not having enough reference sequences for metazoans may

have led to a low number of Polychaeta taxa detected in our eDNA data; there were 148 OTUs assigned as Eukaryota and not any further.

Both nematodes and polychaetes are known to be sensitive to organic enrichment caused by fish farming (Dean, 2008; Mirto et al., 2014; Mirto, La, Gambi, Danovaro, & Mazzola, 2002; Tomassetti & Porrello, 2005). In this study, nematode OTU number correlated much more strongly with pore-water sulphide concentration than polychaete OTU number did, and there were also many more nematode OTUs amplified than polychaete OTUs. This is presumably due to the small size of nematodes (Heip, Vincx, & Vranken, 1985) and that they spend their entire life cycle within sediments, whereas polychaetes are relatively larger-bodied, as well as varying sulphide tolerances and habitat strategies (Fauchald & Rouse, 1997; Pocklington & Wells, 1992). However, the proportion of benthic metazoan reads in our metabarcoding data was not high, which may have impacted the diversity of metazoan species detected. This is a common problem when using a general (i.e. all eukaryotes) *18S rRNA* marker regardless of whether samples are collected in polluted areas (e.g. Lanzén et al., 2016; Lejzerowicz et al., 2015) or non-polluted areas (e.g. Sinniger et al., 2016). Increasing the proportion of metazoan reads should improve the accuracy of eDNA-based methods. Possibilities for achieving this include: treating sediments prior to DNA extraction, such as elutriation by decantation (Brannock & Halanych, 2015) and meiofauna extraction (Fonseca et al., 2011); the use of more metazoan-specific primers; and/or the use of a blocking primer to inhibit amplification of non-target species during PCR (Vestheim & Jarman, 2008). Extracting DNA from a higher amount of sediments also may increase number of species detected.

As already described, we assessed biotic responses to organic enrichment using alpha diversity richness parameters derived from all benthic metazoan reads and, more specifically, from two particular groups: nematodes and polychaetes. In addition, we used eDNA data to investigate responses to organic enrichment at the individual OTU level. Two OTUs showed higher relative abundances in oxic sediments and both of them were assigned to *Sabatieria punctata* . A previous study found the*Sabatieria* genus had higher abundance near the fish cage than at the reference station (Mirto et al., 2002). This discrepancy in results between their findings and this study may be due to a different sample collection period. We collected samples during peak fish production while their monthly sample collection started 15 days after fish were transferred to the cage; populations of benthic fauna can increase when organic enrichment is slight and then decrease when organic enrichment is severe (Pearson & Rosenberg, 1978). We found OTUs assigned to chicken and Atlantic salmon in eDNA data. Chicken feather is used in fish feed diets and sediments were collected from Atlantic salmon farms. We believe they are true reads, although their numbers (9,959 for chicken and 6,371for salmon) were low compared to total number of reads (42.3 million) obtained. Given that these taxa were generally detected close to the net-pens, we feel that these occurrences were directly associated with farming activities.

We did not calculate biotic indices for the sediment samples analyzed here as none is available for this purpose in Canada. While environmental assessments based on geochemistry are very fast to perform, without biological information there is no way for benthic monitoring programs to assess cumulative impacts of fish farming on benthic communities (Lear, Dopheide, Ancion, & Lewis, 2011). Given that eDNA metabarcoding can provide a rapid estimation of species composition and diversity and has high potential to complement current monitoring programs, a few studies have proposed the use of eDNA metabarcoding for benthic environmental health status assessment. For example, Aylagas et al. (2014) used presence-absence of species based on metabarcoding data to calculate a genetics-based AZTI's Marine Biotic Index (gAMBI). However, gAMBI requires OTUs to be accurately assigned to species level, and it uses only species in the AMBI database to calculate the biotic index, potentially rendering most OTUs in a given dataset unusable. Lejzerowicz et al. (2015) used eDNA and eRNA metabarcoding to calculate the Infaunal Trophic Index and three AZTI Marine Biotic Indices, and found that metabarcoding-based indices provided similar ecological assessments of sediments to morphology-based assessments, although only 19.5% of generated sequences were metazoan. Keeley et al. (2018) assigned OTUs from three metabarcoding markers to different Eco-Group categories, and then used OTU presence-absence data to calculate a few biotic indices for benthic impact assessments of fish farms. Those indices were shown to have strong linear relationships with the Enrichment Stage index used for benthic assessments of fish farms in New Zealand (Keeley et al., 2012, 2018). In our study, we

categorized sediments into four organic enrichment statuses based on sediment pore-water sulphide concentration following the Canadian Aquaculture Regulation (AAR, 2016) and used three methods to identify OTUs related to organic enrichment. These OTUs are thus ideal candidates to test for the development of biotic indices to aid benthic impact assessments in British Columbia (and potentially similar environments elsewhere). Alternatively, if further study can confirm the consistency of the responses of these OTUs to organic enrichment status across a higher number of study sites, one or several of them may be amenable for use as a threshold-based bioindicator.

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Data Accessibility

MiSeq sequence data have been deposited in NCBI Sequence Read Archive (SRA) database under the Accession PRJNA474384. DNA barcoding sequence data have been deposited in BOLD database under the Accession CABIB (http://www.boldsystems.org/index.php/MAS_Management_DataConsole?codes=CABIB).

Author contributions

XH conducted eDNA metabarcoding, data analysis, and paper writing. SG collected samples, analyzed morpho-taxonomy and DNA barcoding data in the BOLD database, and contributed to manuscript writing. CA, TS, and JP co-conceived the study. CA led the funding proposal, supervised laboratory aspects of the work, and contributed feedback on data analyses. TS led field-planning and the collection of geochemistry data as well as provided critical input to data analyses. MH provided expertise and laboratory assistance related to the DNA barcoding aspects of the project. KM provided expertise and laboratory assistance related to eDNA metabarcoding. KW provided expertise related to statistical analyses. All authors provided feedback on manuscript drafts.

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