Impact of prawn farming effluent on coral reef water nutrients and microorganisms

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ABSTRACT: Tropical coral reefs are characterized by low-nutrient waters that support oligotrophic picoplankton over a productive benthic ecosystem. Nutrient-rich effluent released from aquaculture facilities into coral reef environments may potentially upset the balance of these ecosystems by altering picoplankton dynamics. In this study, we examined how effluent from a prawn (*Litopenaeus vannamei*) farming facility in Al Lith, Saudi Arabia, impacted the inorganic nutrients and prokaryotic picoplankton community in the waters overlying coral reefs in the Red Sea. Across 24 sites, ranging 0–21 km from the effluent point source, we measured nutrient concentrations, quantified microbial cell abundances, and sequenced bacterial and archaeal small subunit ribosomal RNA (SSU rRNA) genes to examine picoplankton phylogenetic diversity and community composition. Our results demonstrated that sites nearest to the outfall had increased concentrations of phosphate and ammonium and elevated abundances of non-pigmented picoplankton (generally heterotrophic bacteria). Shifts in the composition of the picoplankton community were observed with increasing distance from the effluent canal outfall. Waters within 500 m of the outfall harbored the most distinct picoplanktonic community and contained putative pathogens within the genus *Francisella* and order *Rickettsiales*. While our study suggests that at the time of sampling, the Al Lith aquaculture facility exhibited relatively minor influences on inorganic nutrients and microbial communities, studying the longer-term impacts of the aquaculture effluent on the organisms within the reef will be necessary in order to understand the full extent of the facility's impact on the reef ecosystem.

KEY WORDS: Aquaculture · *Litopenaeus vannamei* · Oligotrophic · Microbial community · Coral reef · SSU rRNA gene · *Francisella* spp.

INTRODUCTION

Aquaculture facilities contribute substantially to the world fish supply and are increasing in abundance worldwide (Naylor et al. 2000). In 2014, over half of the fish that humans consumed were produced through aquaculture, highlighting the importance of aquaculture as a reliable food source for increasing populations (FAO 2016). Aquaculture is important, but the wastes and nutrients produced by these facilities can pose threats to coastal environments (Nogales et al. 2011, Jiang et al. 2013). As a result, monitoring efforts are routinely needed to understand potential impacts. Most aquaculture monitoring efforts are part of required environmental impact assessments that may include collecting measurements of water quality, nutrient concentrations, organic matter outputs, and residual chemicals and bacteria that affect the product quality (Hambrey 2009). In addition, indicator species assays are commonly used to detect a finite number of known pathogens (Hernández et al. 2009, Zhou et al. 2014).

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There has been much less effort towards monitoring how entire bacterial and archaeal communities respond to the influx of aquaculture waste. A more inclusive method that uses comparative phylogenetic analyses to examine how picoplanktonic communities respond can help deepen this understanding.

Microorganisms, including *Bacteria*, *Archaea*, and phytoplankton, are critical to the coastal marine environment and actively participate in biogeochemical cycling of nutrients and organic matter (Kirchman et al. 2007, DeLong 2009). Microbes are sensitive to eutrophication and pollution-based changes in seawater, with some cells exhibiting enhanced or reduced growth under these conditions (dos Santos et al. 2011, Xiong et al. 2015). Studies have shown that in oligotrophic waters, continual enrichment of nitrate and phosphate alters the native bacterial community composition (Chen et al. 2016, Dong et al. 2017). As a result, microorganisms may be able to serve as sensitive indicators of change, especially in oligotrophic environments where the magnitude of nutrient and organic matter inputs from aquaculture could be much higher than natural inputs to the environment. For example, the naturally oligotrophic coral reef waters of the Red Sea feature very low nutrient concentrations of 0.05–0.1 μM phosphate, <0.1 μM ammonium, and <0.3 μM nitrate+nitrite (Furby et al. 2014). Therefore, water-column samples from coral reefs located within the Red Sea are ideal for identifying how anthropogenic inputs from aquaculture facilities perturb the low-nutrient system and impact the bacterial and archaeal picoplankton communities.

Nutrient pollution and eutrophication from aquaculture are important problems affecting tropical coral reef ecosystems that reside in oligotrophic waters. Specifically, aquaculture runoff introduces ammonium, phosphate, and organic solids into the surrounding water. Studies have shown that fish farm runoff can reduce the survivorship of juvenile corals (Villanueva et al. 2005) and impair coral reproduction (Loya et al. 2004), as well as impact coral-associated microbes (Garren et al. 2008, 2009, Campbell et al. 2015). Studies also demonstrate that the planktonic microbial community, which is easier to sample and analyze than coral-associated microbes (e.g. Apprill et al. 2016), shows similar community changes in response to environmental aquaculture pollution (Sousa et al. 2006, Garren et al. 2008, Fodelianakis et al. 2014, Xiong et al. 2015). For example, in aquaculture environments such as a coastal shrimp pond in southeast China (Wei et al. 2009) and a fish farm north of Crete, Greece (Fo-

delianakis et al. 2014), the seawater microbial communities had reduced phylogenetic diversity and experienced overall shifts in community composition. Increases in *Proteobacteria*, free-living bacteria, and virus-like particles were also noted in the oligotrophic reef waters of the Philippines, correlating with organic nutrient enrichment caused by a fish farm (Garren et al. 2008). These often site-specific alterations to the planktonic microbial community by aquaculture speak to the complexity of these oligotrophic ecosystems. It also exemplifies the need for continued assessments of these impacts, especially in understudied areas such as the eastern Red Sea.

The National Aquaculture Group, formerly known as the National Prawn Company, is a coastal aquaculture facility located next to coral reefs in the eastern Red Sea, near the city of Al Lith, Saudi Arabia. It is one of the largest desert aquaculture facilities in the world and covers approximately 250 km². From 2008 to 2010, our team examined the Red Sea marine environment to identify potential sources of putative pathogens that were associated with the presence of harmful coral lesions (Apprill et al. 2013, Furby et al. 2014). As part of this study, we examined whether the effluent from the National Prawn Company aquaculture facility could be a potential source of nutrients and pathogens to the surrounding environment. At the time of the sampling in 2009, the facility was hatching and rearing approximately 15 000 tons of white prawn *Litopenaeus vannamei* annually in ponds covering a total surface area of 2800 ha (https://web.archive.org/web/20091130220933/http://www.robian.com.sa:80/home.html; see also www.naqua.com.sa). Since the time of sampling, the facility expanded to include multiple sea cages and many more shrimp farms and produces upwards of 100 000 tons of marine products annually, including shrimp, sea cucumber, and sea bass (www.naqua.com.sa). Furthermore, Saudi Arabia has plans to expand its aquaculture industry, with multiple investments in new facilities (Mon Chalil 2015). In the context of continuous expansions of the aquaculture industry, it is important to understand the extent to which this industry may threaten the adjacent delicate reef ecosystem. In this study, we aimed to assess the impact of the effluent from this aquaculture facility on the surrounding oligotrophic reef environment by examining the inorganic nutrient concentrations and microbial communities in an area extending nearly 22 km from the outfall. We hypothesized that concentrations of inorganic nitrogen and phosphorus would be elevated at the outfall, a distinct microbial community would reside there, including known
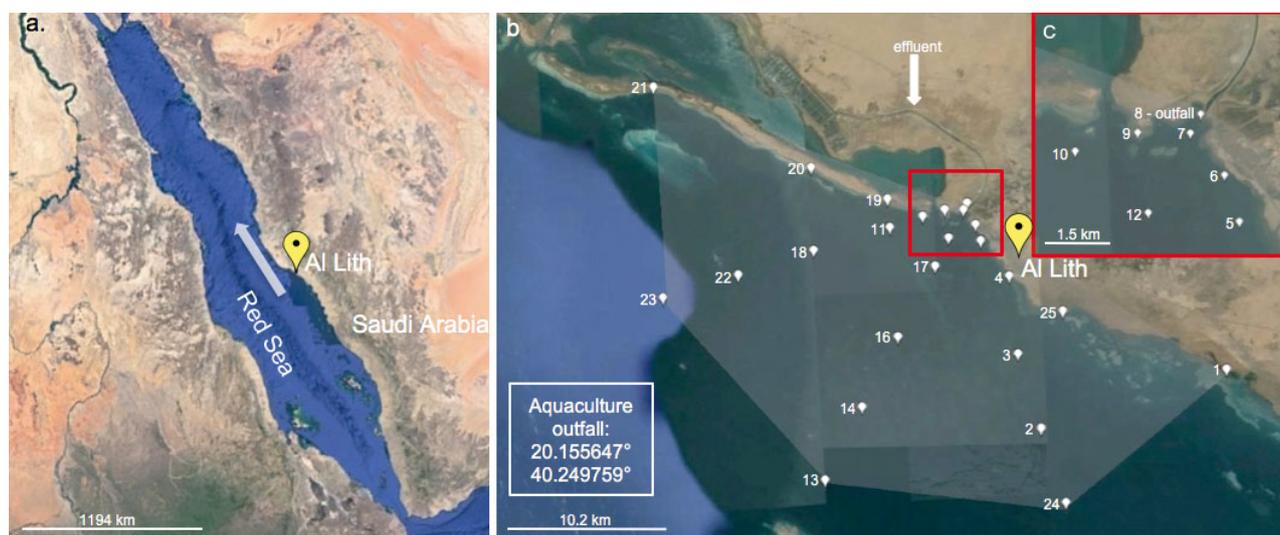
Fig. 1. (a) Location of the aquaculture facility in Al Lith, Saudi Arabia (yellow pin), in the eastern Red Sea. Gray arrow: the prevailing surface currents that move S to N, primarily along the eastern coast. (b) The 24 sites that were sampled along 5 transects extending from the aquaculture outfall up to 21.7 km from the outfall. (c) Inset: a more detailed map of the outfall (Site 8) and nearby sites. Images reproduced according to Google permissions and incorporate data from NOAA, US Navy, National Geospatial-Intelligence Agency, General Bathymetric Chart of the Oceans, DigitalGlobe, and US Geological Survey

pathogens, and that this impact would diminish with increasing distance from the outfall.

MATERIALS AND METHODS

Sampling

Sampling occurred in the eastern Red Sea, surrounding the runoff from the National Prawn Company aquaculture facility in Al Lith, Saudi Arabia (Fig. 1a). Seawater was sampled from 24 stations surrounding the aquaculture facility using a directional-transect grid pattern (Fig. 1b,c) from October 9 to 12, 2009, during 10:00–16:00 h. The sampling pattern extended in 5 transects away from the outfall point source: north, northwest, west, southwest, and south along the coastline (Table A1 in the Appendix). Sampling occurred first at the mouth of the outfall, and then was spaced 0.5–21.7 km along the 5 transects (Fig. 1). Seawater was sampled at a 10 m depth, which was just above the coral reef for the shallower sites, into 20 l acid-washed carboys. Inorganic nutrients and microbial abundance samples were collected and processed as previously outlined in Furby et al. (2014). Briefly, for inorganic nutrients, 150 ml polypropylene acid-washed bottles were filled with seawater and frozen at −20°C. Samples (1 ml) for cell counts were fixed in 1% (v:v) paraformaldehyde (final concentration) and placed in cryovials in liquid

nitrogen for 3 wk, then frozen at −80°C. Seawater for DNA analyses was stored on ice for no more than 4 h before it was filtered onto 0.22 µm Durapore membrane filters (142 mm) (Millipore) with a peristaltic pump. Filters were then frozen in liquid nitrogen.

Nutrient analysis

Nutrients were analyzed as outlined in Furby et al. (2014). In summary, dissolved concentrations of NH_4^+, $NO_3^-+NO_2^-$, NO_2^-, PO_4^{3-}, and silicate were determined with a continuous segmented flow system including a Technicon AutoAnalyzer II (SEAL Analytical) and an Alpkem RFA 300 Rapid Flow Analyzer. $NO_3^-+NO_2^-$ and NO_2^- were measured according to Armstrong et al. (1967). An adjusted molybdenum blue method was used to measure PO_4^{3-} (Bernhardt & Wilhelms 1967). The indophenol blue method was used to measure concentrations of NH_4^+ (USEPA 1983), and validated using a method developed by Holmes et al. (1999).

Direct cell counts

Microbial cell counts were enumerated using the flow cytometry methods described in Furby et al. (2014). To summarize, the preserved samples were analyzed using stained and unstained methods to

determine the number of pigmented and non-pigmented cells within each sample. The aliquots of sample for the unstained method were run on an EPICS ALTRA flow cytometer (Beckman Coulter) to determine abundance of *Cyanobacteria* (*Prochlorococcus* and *Synechococcus*) and small eukaryotic phytoplankton (picoeukaryotes). The aliquot of sample for staining was prepared by diluting the sample 1:10 into a 30 mM (final concentration) potassium citrate buffer solution, and staining with Sybr Green I (1:5000 final dilution of initial stock) (Molecular Probes) for a duration of 2 h in the dark at 4°C. Excitation of the fluorescent stain was accomplished using a laser operating at a wavelength of 488 nm on the same EPICS ALTRA flow cytometer, and this allowed for enumerating picoplankton (*Bacteria* and *Archaea*) on the basis of DNA staining (Sybr Green I green fluorescence), chlorophyll, phycoerythrin, forward scatter, and 90° side scatter signatures. *Prochlorococcus* cell counts from the unstained method were subtracted from total prokaryotic cells to obtain counts of non-pigmented picoplankton. FlowJo software (v.6.3.3, Tree Star) was used for off-line data analysis.

DNA analysis

The filters were cut into quarters, and DNA was extracted from one of the quarters using a bead beating method combined with a sucrose-lysis extraction and spin-column separation. Briefly, 0.1 mm glass beads were used to homogenize cellular biomass collected on the filters for 10 min in a solution of 875 µl sucrose-EDTA lysis buffer (0.75 M sucrose, 20 mM EDTA, 400 mM NaCl, 50 mM Tris) and 100 ml of 10% sodium dodecyl sulfate. This was followed by a Proteinase K digestion at 55°C for 4 h, and separation of the DNA using the spin columns supplied by the DNeasy kit (Qiagen) (Santoro et al. 2010). The DNA samples were individually amplified with the 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806RB primers (5′-GGA CTA CNV GGG TWT CTA AT-3′), with the modified reverse primer designed to enhance the detection of different SAR11 clades (Apprill et al. 2015). The primers were designed similar to Kozich et al. (2013) and each included a unique 8 bp barcode, 10 bp pad, and 2 bp link in addition to the primers described above. PCR reactions were carried out in a Bio-Rad thermocycler using triplicate 25 µl volumes for each sample. Each reaction included 1.25 U GoTaq Flexi DNA Polymerase (Promega), 5× Colorless GoTaq Flexi Buffer, 2.5 mM

MgCl$_2$, 200 µM dNTP mix (Promega), 200 nM of the corresponding barcoded primer, and 1–4 ng of genomic DNA template. The reaction conditions were as follows: initial denaturation for 2 min at 95°C, followed by an iteration of 20 s at 95°C, 15 s at 55°C and 5 min at 72°C for 25–28 cycles, and a final extension step for 10 min at 72°C. Five µl of the reaction products were run on a 1% agarose/TBE gel containing the HyperLadder 50 bp DNA ladder (generally 5 ng µl^{-1}) (Bioline). After pooling the triplicate reactions per sample, the PCR products were purified with the QIAquick Purification Kit (Qiagen). The samples were quantified using the Qubit 2.0 Fluorometer with the dsDNA High Sensitivity Assay (Life Technologies). The PCR products were then combined into equimolar ratios and processed for sequencing using 250 bp paired-end MiSeq (Illumina) at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois. Raw sequence data were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession number PRJNA357506.

Sequence analysis

Sequence data were filtered and analyzed using mothur v.1.36.1 (Schloss et al. 2009). This included constructing contigs, filtering out long amplicons (over 255 bp), and removing chimeras detected using de novo UCHIME v.4.2.40 (Edgar et al. 2011). The taxonomic assignment for each sequence was performed in mothur using the SILVA SSU reference database (v.123) and the *k*-nearest neighbor algorithm with an 80% cutoff. The number of small subunit ribosomal RNA (SSU rRNA) sequences per sample varied between 266 and 55 182 and the dataset was therefore subsampled to a depth of 10 200 sequences per sample, resulting in the loss of only Sample 20 (Appendix 1). Operational taxonomic units (OTUs) for each sample were generated using the minimum entropy decomposition (MED) clustering algorithm (Eren et al. 2015). MED further reduced the sequence reads to between 8853 and 9538 sequences per sample (Appendix 1).

Graphical and statistical analyses

Ocean Data View software (v.4.7.8, 64 bit) (Schlitzer 2002) was used to create contour plots of nutrients and microbial abundances using data-interpolating variational analysis (DIVA) gridding (40 × 40

scale length). PRIMER-E 7 (v.7.0.11, Quest Research) was used to compare the composition of OTUs at each sampling site. The data were square-root transformed, compared using Bray-Curtis similarity, and plotted using non-metric multidimensional scaling (nMDS) to compare microbial community structure across samples. Information regarding proximity to the outfall was superimposed onto the symbols for each sample in the nMDS plot. In addition, bar plots were generated in Excel for Mac 2011 (v.14.6.9, Microsoft) to illustrate microbial community composition by plotting the relative percent abundance of taxonomic groups of *Bacteria* and total *Archaea*.

Alpha diversity metrics of microbial communities at each site were computed using the plot_richness function in the phyloseq (v.1.16.2) and ggplot2 (v.2.1.0) R packages in RStudio (v.0.99.902) (Wickham 2009, McMurdie & Holmes 2013). Specific metrics plotted include 'observed' (observed richness), 'Chao1' (estimator of richness), and 'invsimpson' (inverse Simpson index of diversity). Differentially abundant OTUs between the outfall and other sites were obtained using DESeq2 (v.1.12.4) in RStudio (Love et al. 2014). Specifically, OTU sequence count data was compared between the outfall site and 6 different distance groups away from the outfall that encompassed the other sites (0.5–1.5, 2.5–3, 4.5–5.3, 9–10.1, 14.5–15.2, and 20–21.7 km). Significant differential abundance was determined in DESeq2 using the adjusted p-value (p < 0.05). Box plots of relative abundance data for significantly enriched (at the outfall) OTUs were created in PRIMER-E 7 (v.7.0.11).

RESULTS

Nutrients

Nutrient concentrations were generally similar across most of the sites spanning 0–21.7 km from the outfall (Fig. 2), with the exception of ammonium and phosphate, which were elevated at the outfall by 5–75 times and 4–14 times, respectively, compared to the other sites (Fig. 2a,d). Ammonium was 0.60 µM at the outfall and below 0.12 µM (Site 19) at all other sites (Fig. 2a,j). The concentration of phosphate at the outfall was the highest measured in the study, at 0.67 µM, while all other sites were lower, below 0.17 µM (Site 7) (Fig. 2d,m). Concentrations of nitrite, nitrate+nitrite, and silicate were not elevated near the outfall, and were similar in concentration to the more distant sites (Fig. 2b,c,e,k,l,n). Over the study

area, the combined nitrate+nitrite concentrations ranged from undetectable to 0.87 µM, nitrite ranged from 0.02 to 0.11 µM, and silicate ranged from 0.08 to 1.64 µM (Fig. 2b,c,e).

Microbial abundances

Cell counts of microbial groups were measured at all 24 sites (Fig. 2f–i), and there were no clear patterns in cell abundances, either elevated or depleted, directly at the outfall. Concentrations of non-pigmented picoplankton, a proxy for heterotrophic *Bacteria*, *Archaea*, and non-pigmented photoheterotrophic *Bacteria*, were high at the outfall (1.5×10^6 cells ml^{-1}) as well as at Sites 19, 17, 10, and 11 (1.3 to 1.5×10^6 cells ml^{-1}), which are all between 3 and 5 km away from the outfall (Fig. 2f,o). Concentrations were up to 9.3×10^5 cells ml^{-1} lower at the remaining sites, which ranged from 5.8×10^5 to 1.2×10^6 cells ml^{-1}. *Synechococcus* spp. were depleted directly at the outfall (1.5×10^5 cells ml^{-1}) compared to nearby Sites 7 and 9 that are ≤1.5 km away ($2.0–2.1 \times 10^5$ cells ml^{-1}) (Fig. 2q). *Synechococcus* spp. were most abundant at sites 2.5 km from the outfall (Sites 5 and 12; $3.0–3.2 \times 10^5$ cells ml^{-1}; Fig. 2q). Abundances of *Prochlorococcus* spp. varied from 2.4×10^4 to 6.4×10^4 cells ml^{-1}, with no major changes observed over the sampling area (Fig. 2g). The abundance of picoeukaryotes ranged from 2.1×10^3 to 1.2×10^4 cells ml^{-1} (mean: 4.6×10^3 cells ml^{-1}), and were also consistent across the sampling area (Fig. 2i).

Microbial community phylogenetic diversity

Alpha diversity indices of bacterial and archaeal OTUs based on SSU rRNA gene sequences showed some variability between the sites. The observed community richness, or number of observed OTUs, ranged between 225 and 357 OTUs, and richness at the outfall site fell near the middle of this range, at 291 (Fig. 3a). Two sites, Site 25 (9 km from outfall) and Site 6 (1.5 km from outfall), contained microbial communities of lower richness, at 225 and 240 OTUs, respectively. The Chao1 estimator of richness produced patterns similar to the observed richness (Fig. 3a,b), indicating that the depth of sequencing utilized was appropriate for the study. When the samples were organized by distance from the outfall, there appeared to be a potential increase in estimated richness with increasing distance to ~5 km. Again, Sites 6 and 25 had lower predicted richness
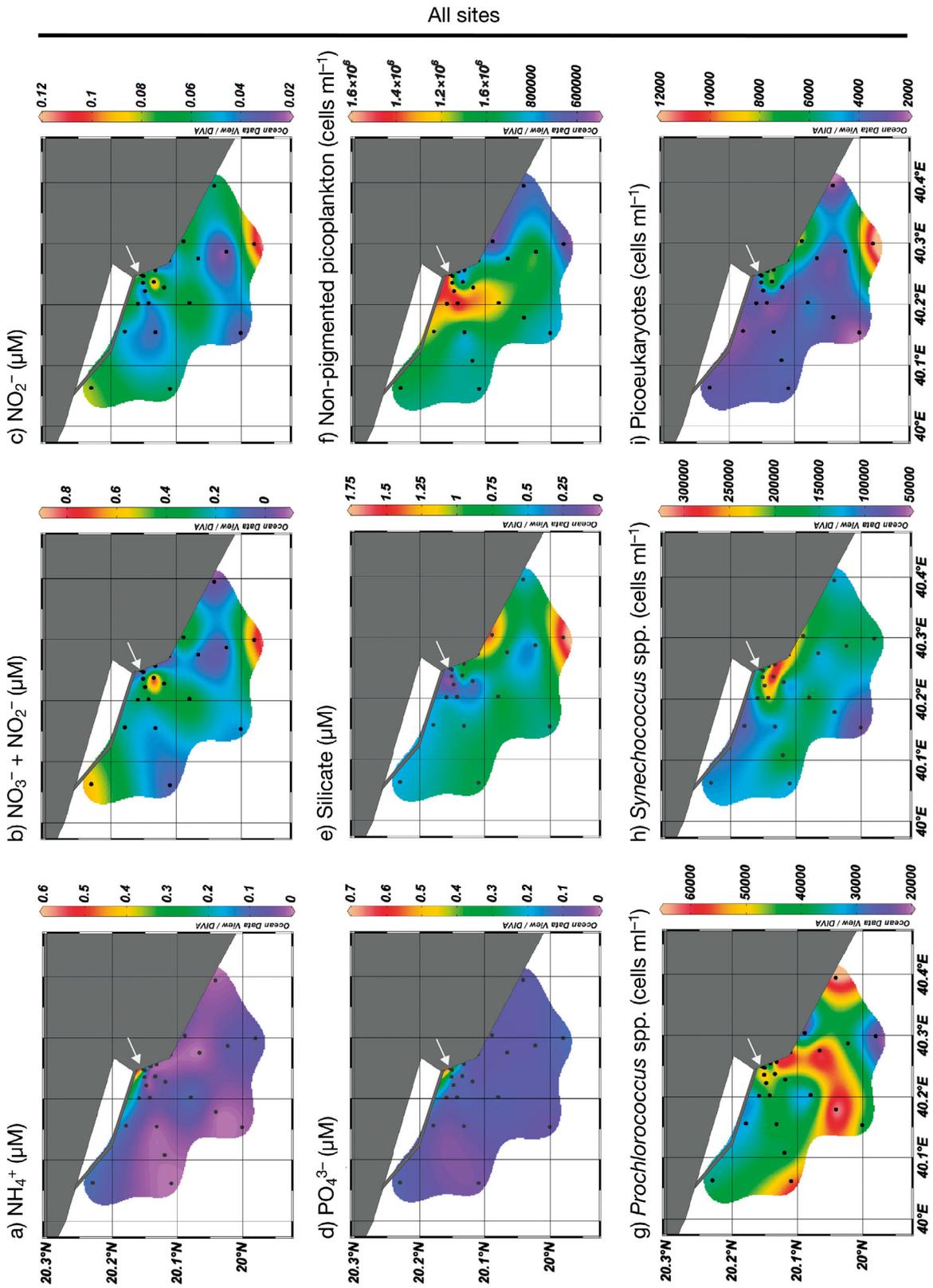
Fig. 2. Maps of surface concentrations of dissolved inorganic nutrients and microbial abundances at (a−i) all sample sites, and (j−r) sites <5.3 km from the aquaculture outfall. Inorganic nutrients: (a,j) NH_4^+, (b,k) $NO_3^- + NO_2^-$, (c,l) NO_2^-, (d,m) PO_4^{3-}, and (e,n) silicate. Microbial abundances: (f,o) non-pigmented picoplankton, (g,p) *Prochlorococcus* spp., (h,q) *Synechococcus* spp., and (i,r) picoeukaryotes. White arrows: the outfall site. Northern coastline has been drawn in, to more accurately depict the geography
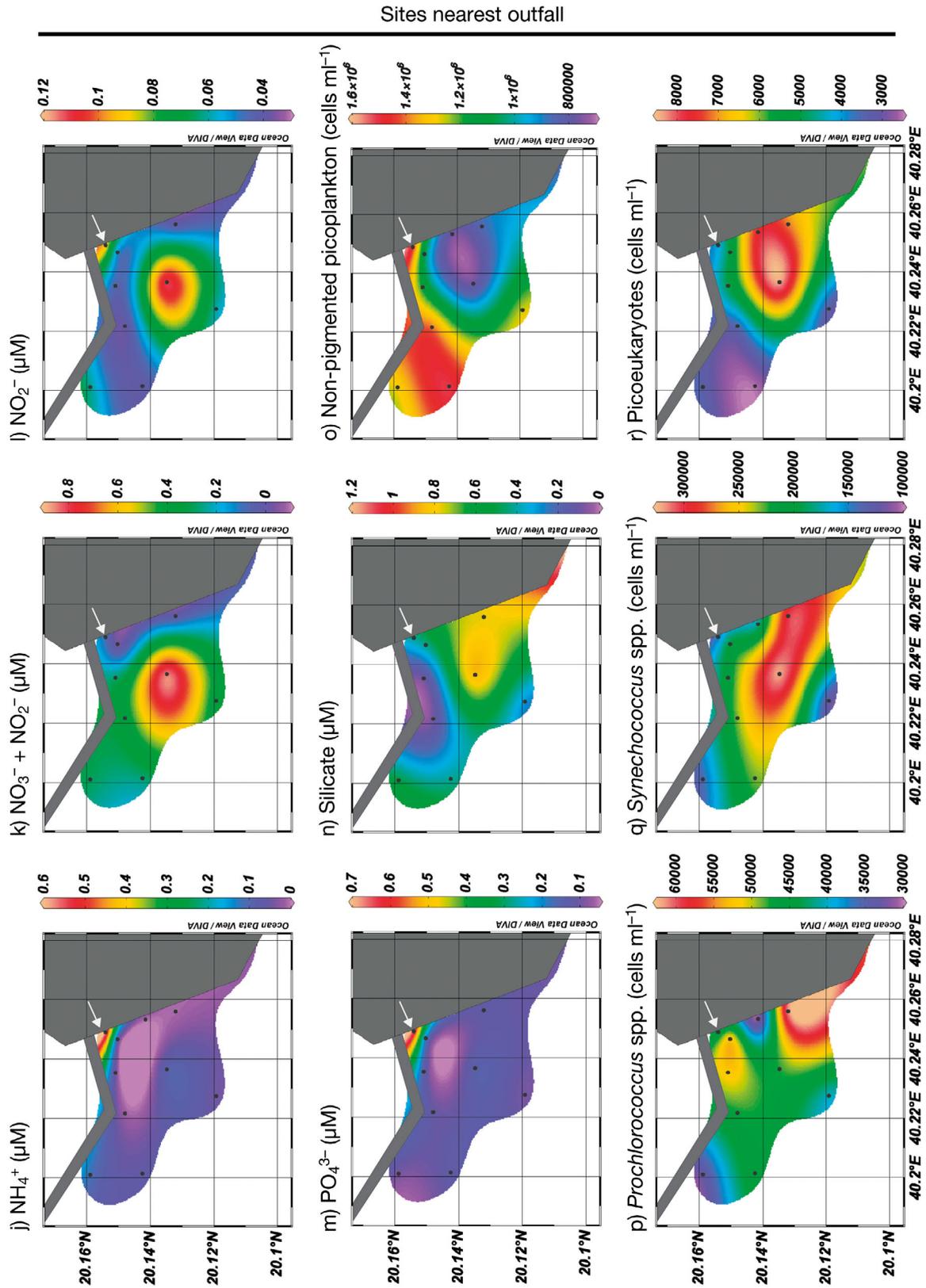
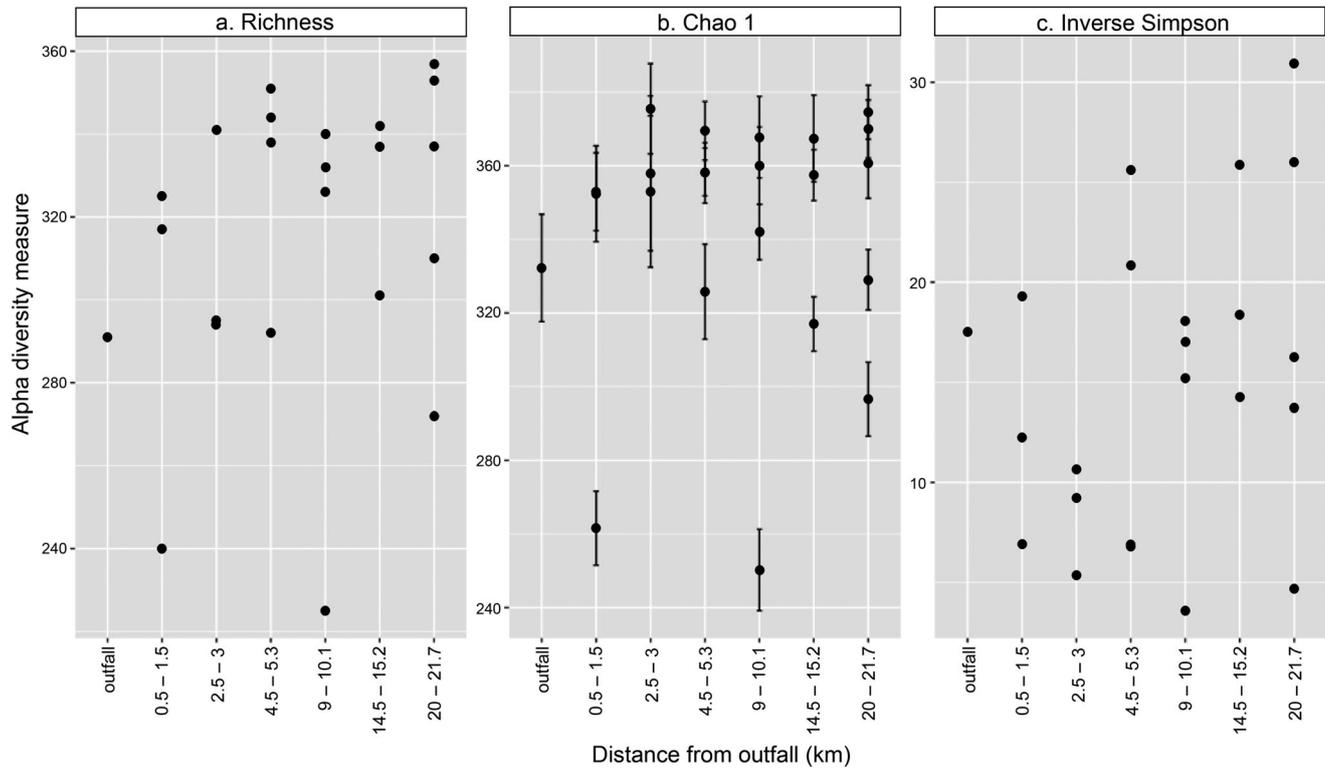Sites nearest outfall

Fig. 2 (continued)

Fig. 3. Measures of alpha diversity based on bacterial and archaeal small subunit ribosomal RNA (SSU rRNA) genes at each sampling site: (a) community richness (e.g. number of operational taxonomic units), (b) Chao1 estimator (bars: 95% confidence intervals), and (c) inverse Simpson index. Data are organized into groups based on distance from the outfall

(Fig. 3b). The inverse Simpson index of diversity also indicates community evenness (Fig. 3c), and it ranged from low diversity and evenness, at 3.58 (Site 25, 9 km from the outfall), to higher diversity and evenness, at 31 (Site 13, 20 km from the outfall), with a mean of 15. Even though Site 6 was low in both observed and estimated richness (Fig. 3a,b), it was not low in diversity relative to the other sites, indicating that Site 6 had a more even microbial community. In terms of diversity, the outfall, with an inverse Simpson index of 17.5, was similar to the other sites.

Microbial community composition

The SSU rRNA gene sequences were dominated by *Cyanobacteria* and *Alphaproteobacteria* and showed similar trends in composition across the sites (Fig. 4). Two sites, 24 and 25, were made up of mostly *Cyanobacteria*, comprising 60 and 75% of total sequences, respectively. The outfall (Site 8) was similar to most sites, except that it had 3 times as much *Flavobacteria* as all other sites (15.6% compared to a mean of 4.7% for the other sites) (Fig. 4). An nMDS analysis provided a spatial representation of commu-

nity composition resemblance between sites (Fig. 5). Again, in this analysis, the southern Sites 24 and 25 were separated from the group, supporting how microbial communities at these sites were distinct from most of the other sites. Coastal Site 6 was also separated, both from the entire group of sites, and also from the other sites 0.5–1.5 km (Fig. 5) from the outfall. In general, the arrangement of sites in the nMDS plot corresponds to actual distance from outfall, demonstrating that the bacterial and archaeal communities shifted in composition with increasing distance away from the effluent outfall. The 0.5–3 km group plotted closer to the outfall than the 4.5–10.1 km distance group. In addition, the 14.5–21.7 km group was even farther from the outfall point in the nMDS plot (Fig. 5). The outfall site is distinctly separate in the plot, indicating that there are taxa within the community that are unique to the outfall.

Differentially abundant outfall bacteria

Microbes indicative of the outfall effluent were examined using the SSU rRNA gene sequence count data, and 22 OTUs were significantly elevated or de-
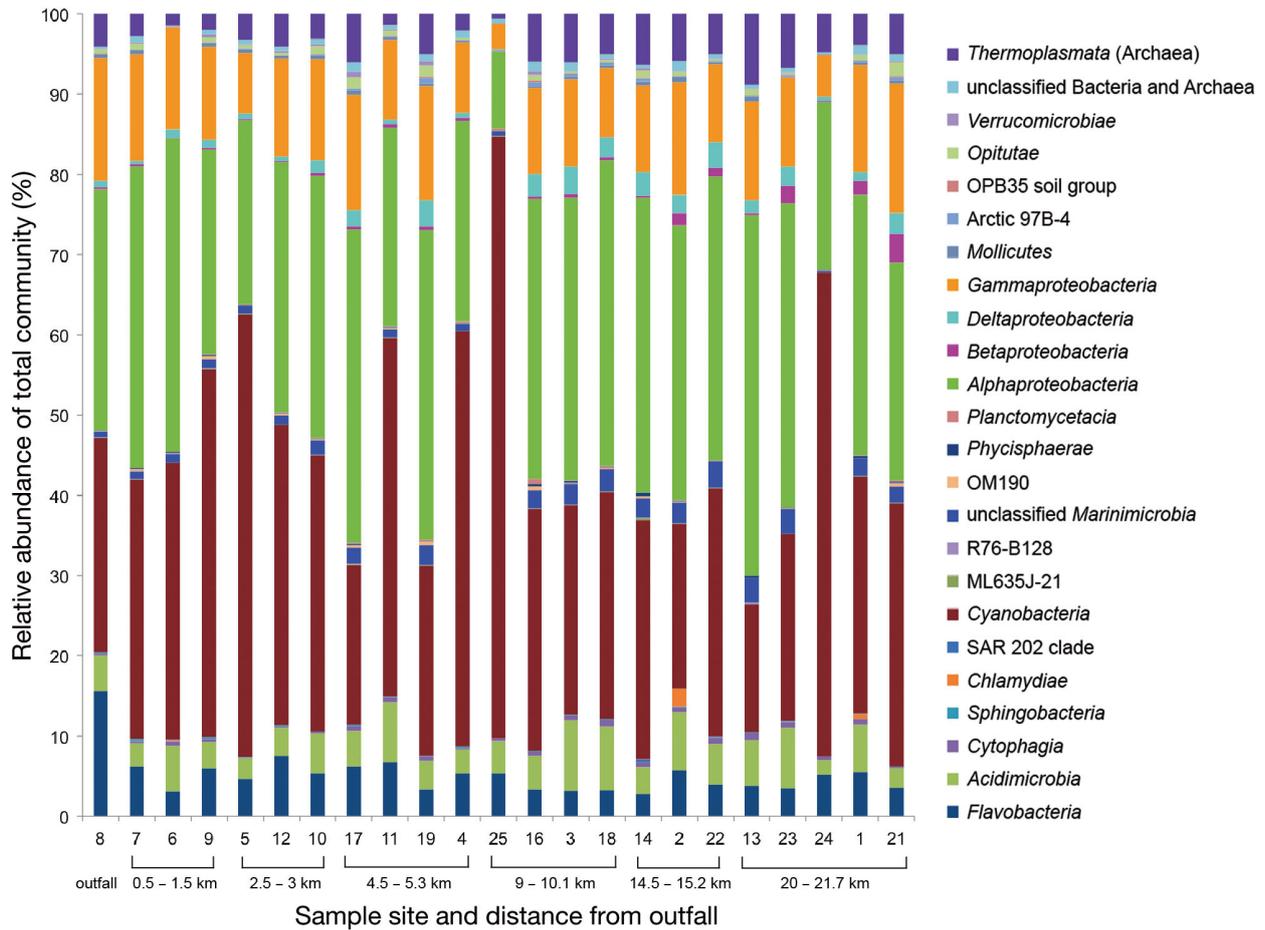
Fig. 4. Relative abundances of small subunit ribosomal RNA (SSU rRNA) gene sequences organized by taxonomic class for each site. Sites are organized by distance from outfall, with Sample 8 (outfall) on the far left and Sample 21 (21.7 km from Site 8) on the far right

pleted at the outfall when compared to the 6 distance groupings (0.5–1.5, 2.5–3, 4.5–5.3, 9–10.1, 14.5–15.2, and 20–21.7 km) (Fig. 6, Table 1). Of these, 7 OTUs were significantly elevated at the outfall (Fig. 6). These included sequences classifying as *Francisella* spp., *Caedibacter* spp., uncultured *Gammaproteobacteria*, members of the NS4 Marine Group, uncultured *Rickettsiales*, uncultured *Flavobacteriaceae*, and uncultured *Cryomorphaceae* (Fig. 6a–g). Sequences of those OTUs enriched at the outfall were found in lower abundances at the more distant site groupings, and were significantly different at many of these groups (p < 0.05; Fig. 6). For OTUs 622 (*Francisella* spp.), 705 (*Caedibacter* spp.), and 1653 (uncultured *Gammaproteobacteria*), sequence counts were significantly lower at all distances compared to the outfall (Fig. 6a–c). Sequence counts for OTUs 1743 (NS4 Marine Group) and 1342 (uncultured *Rickettsiales*) were significantly lower at all distance groups except for the closest one, 0.5–1 km from the outfall
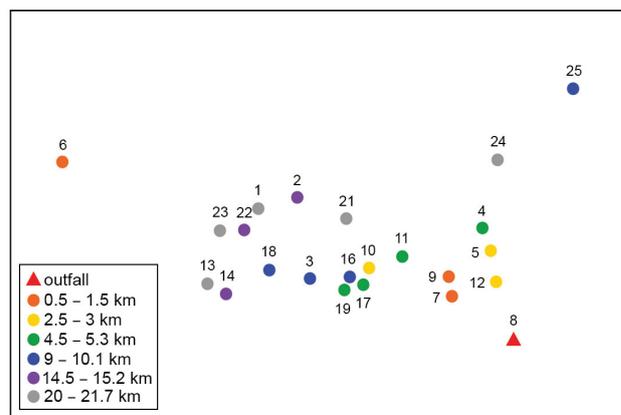

Fig. 5. Non-metric multidimensional scaling analysis of bacterial and archaeal community composition at each study site based on small subunit ribosomal RNA (SSU rRNA) gene sequences. Relative abundance data were square-root transformed and the resemblance of community composition was determined using Bray-Curtis similarity. Sites are grouped by distance from the outfall and the numbers indicate site. The 2-D stress of the solution is 0.06
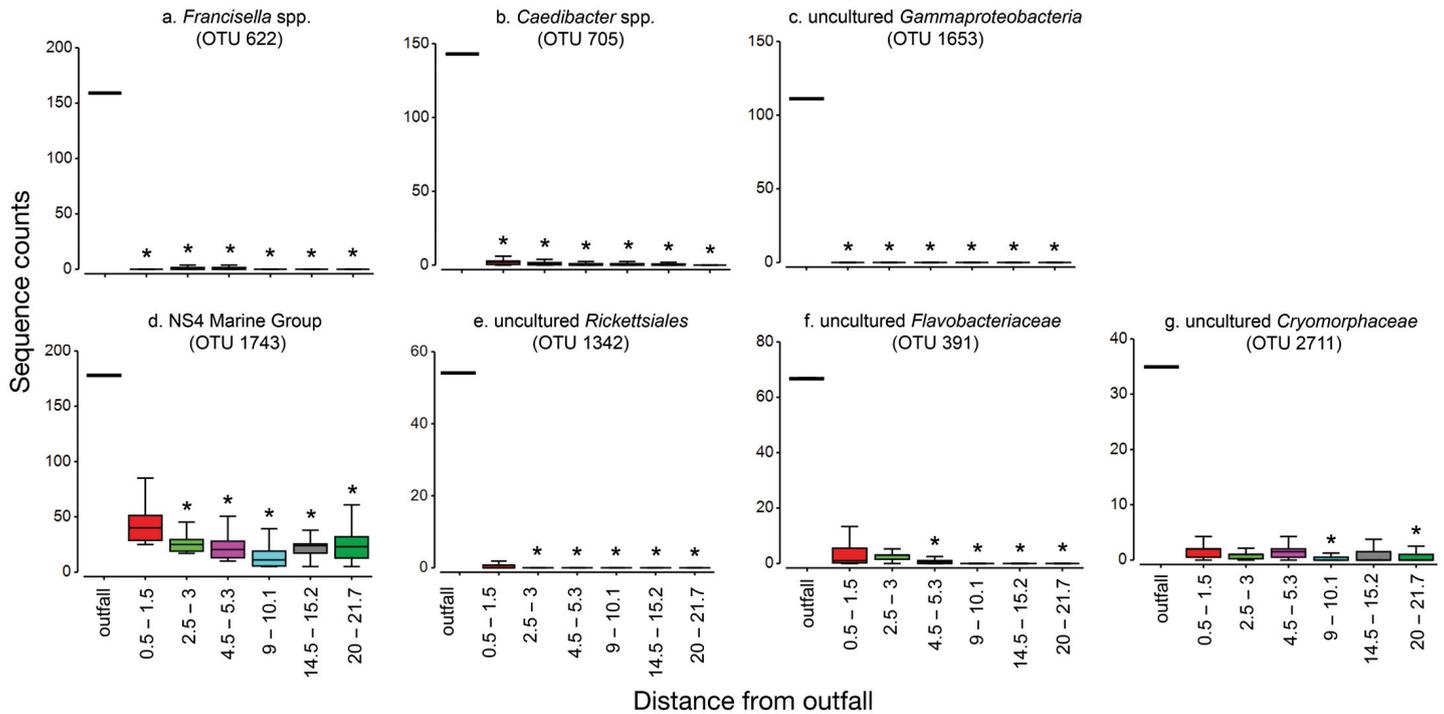
Fig. 6. Box-and-whisker plots of sequence counts for the 7 operational taxonomic units (OTUs) that were significantly enriched at the outfall site: (a) *Francisella* spp., (b) *Caedibacter* spp., (c) uncultured *Gammaproteobacteria*, (d) NS4 Marine Group, (e) uncultured *Rickettsiales*, (f) uncultured *Flavobacteriaceae*, and (g) uncultured *Cryomorphaceae*. *Distance groups with significantly depleted sequence counts compared to sequence counts at the outfall (p < 0.05). The outfall includes 1 sample (indicated by single line) and the other distance groups include 3–5 samples. In each plot, middle line denotes median, box outlines upper and lower 25% quartiles, and whiskers denote maximum and minimum values

(Fig. 6d,e). Sequence counts for OTU 391 (uncultured *Flavobacteriaceae*) were significantly different from the outfall for the 4 farthest distance groups (4.5–5.3, 9–10.1, 14.5–15.2, and 20–21.7 km), but not the 2 closest distance groups (Fig. 6f). Sequence counts for OTU 2711 (uncultured *Cryomorphaceae*) were significantly distinct from the outfall at 2 of the farther distance groups (9–10.1 and 20–21.7 km) (Fig. 6g). The other 15 significantly differentially abundant OTUs were significantly enriched or depleted in 3 or fewer distant groups, when compared to the outfall, as indicated by the positive and negative log2 fold change (Table 1). Out of the 15 significantly enriched OTUs, 11 were from the taxonomic class *Flavobacteria* (Table 1), the class of *Bacteria* found at higher relative abundance at the outfall compared to the other sites (Fig. 4).

DISCUSSION

This study analyzes multiple factors, including nutrient concentrations, microbial cell abundances, and the fine-scale taxonomy and composition of the bacterial and archaeal seawater communities, to examine the impact of an effluent point source from a large aquaculture facility on oligotrophic coral reefs within the Red Sea. Although our results represent data from 2009 and were collected prior to recent expansions of the facility, they show a distinct elevation of ammonium and phosphate and an altered microbial community structure at the outfall, with subtler differences within microbial community composition as distance from the outfall increases.

The elevated levels of ammonium and phosphate at the outfall site were consistent with findings of aquaculture impacts in other studies. Biao et al. (2004) found elevated inorganic nutrient concentrations, including ammonium and phosphorus, in a shrimp farm outlet creek that flowed from shrimp ponds to the Yellow Sea. In that study, changes in ammonium reached 4.7 µM, much greater than in our study, whereas the magnitude of change in inorganic phosphorus concentrations was 0.26 µM, lower than that measured in our study (0.5 µM). In a study of fish cages in the eutrophic environment of the Xiangshan Bay, China, Xiong et al. (2015) observed overall increases in the concentrations of dissolved

Table 1. Operational taxonomic units (OTUs) that were significantly enriched or depleted at the outfall compared to the other distance groups, based on DESeq2 results

OTU	Classification	log2 fold change[a]	Enriched or depleted at outfall	Significant at distance groups (km) (p < 0.05)
622	Gammaproteobacteria, Francisella spp.	8.8; 6.6; 6.6; 9.2; 9.0; 9.5	Enriched	0.5–1.5; 2.5–3; 4.5–5.3; 9–10.1; 14.5–15.2; 20–21.7
705	Gammaproteobacteria, uncultured Caedibacter spp.	5.5; 6.2; 7.1; 7.2; 7.5; 9.4	Enriched	0.5–1.5; 2.5–3; 4.5–5.3; 9–10.1; 14.5–15.2; 20–21.7
1653	Uncultured Gammaproteobacteria	9.1; 9.2; 9.4; 9.4; 9.3; 9.6	Enriched	0.5–1.5; 2.5–3; 4.5–5.3; 9–10.1; 14.5–15.2; 20–21.7
1743	Flavobacteria, NS4 Marine Group	2.9; 3.3; 4.0; 3.3; 3.2	Enriched	2.5–3; 4.5–5.3; 9–10.1; 14.5–15.2; 20–21.7
1342	Alphaproteobacteria, uncultured Rickettsiales	7.7; 8.0; 8.2; 8.2; 8.2	Enriched	2.5–3; 4.5–5.3; 9–10.1; 14.5–15.2; 20–21.7
391	Flavobacteria, uncultured Flavobacteria, Flavobacteriaceae	6.2; 8.2; 8.0; 8.5	Enriched	4.5–5.3; 9–10.1; 14.5–15.2; 20–21.7
2711	Flavobacteria, uncultured Cryomorphaceae	6.1; 5.7	Enriched	9–10.1; 20–21.7
2731	Flavobacteria, uncultured Cryomorphaceae	7.5	Enriched	14.5–15.2
1477	Flavobacteria, uncultured Flavobacteriaceae	6.7	Enriched	14.5–15.2
491	Flavobacteria, NS2b Marine Group	2.2	Enriched	14.5–15.2
2649	Flavobacteria, NS4 Marine Group	3.3	Enriched	14.5–15.2
1744	Flavobacteria, NS4 Marine Group	2.9	Enriched	14.5–15.2
1807	Flavobacteria, NS5 Marine Group	5.6	Enriched	14.5–15.2
2322	Flavobacteria, NS9 Marine Group	6.2	Enriched	14.5–15.2
2329	Flavobacteria, NS9 Marine Group	5.5	Enriched	14.5–15.2
1618	Gammaproteobacteria, Alteromonas spp.	−4.9; −3.7; −4.7	Depleted	0.5–1.5; 14.5–15.2; 20–21.7
2157	Cyanobacteria, uncultured Cyanobacteria	−6.5; −4.8	Depleted	0.5–1; 14.5–15.2
2055	Alphaproteobacteria, SAR116 clade	−3.2	Depleted	14.5–15.2
1018	Alphaproteobacteria, SAR116 clade	−4.7	Depleted	14.5–15.2
789	Alphaproteobacteria, SAR116 clade	−3.8	Depleted	14.5–15.2
2384	Euryarchaeota, Marine Group II	−4.4	Depleted	14.5–15.2
2765	Euryarchaeota, Marine Group III	−4.7	Depleted	14.5–15.2

[a]Order of values corresponds with respective distance group in last column

inorganic nitrogen and phosphate at fish cages relative to a reference site that was 8 km away. The magnitude of increase of dissolved inorganic nitrogen was approximately 2.4 µM, much higher than any concentration measured in our study. In contrast, while overall phosphate concentrations were higher within the eutrophic bay, the increase in phosphate at the fish farm (0.1 µM) was lower than the increase measured in our study (0.5 µM). Although the magnitude of changes varied among studies, the ubiquitous increase in seawater nutrient concentrations in areas supporting aquaculture facilities suggests that they can be a source of nutrient enrichment to both eutrophic and oligotrophic environments. This comparison further suggests that the elevated nutrient concentrations may be attributed to animal waste products generated at the aquaculture facility. Since the time of sampling in 2009, the Al Lith aquaculture facility has expanded considerably to include more shrimp farms and sea cages. If these inorganic nutrient concentrations were among the highest recorded in the Red Sea in 2009, we would hypothesize that nutrient levels will have only increased as a result of the expansions, further threatening the delicate reef ecosystem.

In the study presented here, we found it surprising that the effluent nutrient signal diminished so rapidly just 500 m away from the outfall. This could be related to the oligotrophic nature of the Red Sea waters, in which generally low-concentration or limiting nutrients are quickly mixed and diluted into the surrounding waters, or are rapidly assimilated by bacteria and other planktonic organisms that recycle these nutrients and incorporate them into microbial biomass. Although detailed circulation data for the Red Sea is scant, the study area features a buoyancy-driven boundary current that moves

generally from south to north, which may also be contributing to the incorporation of nutrients into the surrounding waters (Eshel & Naik 1997). Another factor may be the length of the effluent canal. Biao et al. (2004) found diminishing concentrations of all measured inorganic nutrients along an outlet canal that extended from 3 shrimp ponds to the Yellow Sea, over a distance of 11 km. The effluent canal of the National Prawn Company facility runs the length of the shrimp pond area, over 20 km long, and may be acting as a potential biological buffer where higher levels of nutrients are consumed before reaching the oligotrophic waters.

Of the microbial cells enumerated, non-pigmented picoplankton were the only cell groups found to be elevated at the outfall, as well as at nearby sites. Non-pigmented picoplankton are primarily heterotrophic *Bacteria* and *Archaea* as well as non-pigmented photoheterotrophic *Bacteria*, and their abundance is often related to the organic carbon availability (Jumars et al. 1989), which was likely elevated as a result of shrimp organic wastes in the effluent. Although organic carbon was not measured here, preventing a complete assessment, a study of bacterial communities below a fish farm cage found increased abundances of heterotrophic bacteria as a result of total organic matter enrichment (La Rosa et al. 2004). This suggests the elevated abundances of non-pigmented picoplankton at the outfall may be due to populations of these cells residing in the organic-rich effluent, likely confirming the presence of increased organic matter. Indeed, future studies of aquaculture effluent should include quantification of particulate and dissolved organic carbon.

One particular strength of this study was the examination of the microbial SSU rRNA sequences from general bacterial and archaeal primers, which allowed for a more holistic examination of the communities compared to cultivation-dependent or indicator microbial assays, such as loop-mediated isothermal amplification assays that detect known bacterial pathogens (Zhou et al. 2014). An example of one of the benefits of using cultivation-independent techniques to study microbial communities within the environment is that it allowed us to produce new knowledge of potential indicator bacterial taxa that may be originating from shrimp aquaculture. We found alterations in the entire microbial community composition with increasing distance away from the aquaculture effluent point source. This finding is consistent with studies that found changes in the microbial communities between treated wastewater runoff and the seawater surrounding coral reefs in

Florida (Campbell et al. 2015) and in the Red Sea (Ziegler et al. 2016). It also confirms other study results that found alterations in bacterial communities in oligotrophic areas subject to aquaculture (fish farm) effluent (Garren et al. 2008, Fodelianakis et al. 2014). In our study, 22 groups of bacteria were identified as being significantly elevated or depleted at the outfall site. Of these, 7 were elevated at the outfall of the effluent canal when compared to multiple distances, suggesting that they potentially originated at the aquaculture facility. Of these enriched groups, 2 OTUs, classifying as *Francisella* spp. and uncultured *Rickettsiales*, are related to known pathogens. *Francisella* spp. have been implicated as pathogens for a wide taxonomic diversity of mammals and fish, as well as free-living cells (Sjödin et al. 2012). Pathogenic *Francisella* generally target marine organisms, causing disease in cod (Olsen et al. 2006), tilapia (Mauel et al. 2007), and giant abalone (Kamaishi et al. 2010). In addition, *Francisella* spp. can also impact a wide diversity of land mammals, including humans, through arthropod vectors or infected water (Pérez et al. 2016). It is likely that the infection breadth of *Francisella* spp. is highly underestimated (Birkbeck et al. 2011), and the significant occurrence of *Francisella* spp. at the shrimp aquaculture outfall may suggest that *Francisella* spp. originated in the shrimp ponds. Some pathogenic species, including *F. noatunensis* (cod pathogen) and *F. tularensis* (human pathogen), likely persist poorly in the environment without a host (Duodu & Colquhoun 2010). Despite this, *Francisella* spp. have been isolated in seawater samples from around the world (Barns et al. 2005, Berrada & Telford 2010, Duodu et al. 2012). Some pathogenic *Francisella* strains may interact with single-celled eukaryotes, allowing them to persist successfully in the environment (Verhoeven et al. 2010). While presence and abundance of single-celled eukaryotes were not measured in this study, they generally feed on bacteria, and increased levels of heterotrophic bacteria at the effluent outfall may have provided a favorable environment for eukaryotic *Francisella* spp. hosts. The other putative pathogen detected in this study was uncultured *Rickettsiales.* While not every *Rickettsiales* bacterium is pathogenic, these bacteria belong to an order that is characterized by having both obligate intracellular and free-living lifestyles. *Rickettsiales* can invade arthropod hosts (Darby et al. 2007), and in fact, the first marine *Rickettsiales* pathogen to be characterized, 'Candidatus Hepatobacter penaei', was originally isolated from *Penaeus vannamei*, and subsequently found in both an arthropod and crustacean

as well as a commonly cultured shrimp in the western hemisphere (Nunan et al. 2013). *Caedibacter* spp. encompass another interesting group of bacteria elevated at the outfall. While this bacterium is not pathogenic, this genus comprises species that are unique endosymbionts of paramecia (Beier et al. 2002). Paramecia have been isolated from fresh, brackish, and seawater environments where they can prey on other bacteria and algae (Fokin et al. 1999, Fokin 2010). While little information on specific associations of paramecia within shrimp aquaculture exist, the outfall of the effluent canal was characterized by increased counts of heterotrophic bacteria, and may have hosted an environment favorable to the algae- and bacteria-consuming paramecia, which could account for the increase in *Caedibacter* spp. at the outfall.

Other bacteria elevated at the outfall included common seawater bacteria, including uncultured *Gammaproteobacteria*, uncultured *Flavobacteriaceae*, NS4 Marine Group, and uncultured *Cryomorphaceae*. The *Flavobacteriaceae* are a large family of marine bacteria, and this family includes some species that are associated with phosphorus and nitrogen enrichment as well as shrimp aquaculture (Maeda et al. 2002, Abell & Bowman 2005). NS4 Marine Group and uncultured *Cryomorphaceae* are also common bacteria in seawater, but less is known about their physiology (Alonso et al. 2007, Bowman 2014). While some bacteria were found at higher abundances at the outfall, others were significantly depleted at the outfall. For example, 3 OTUs belonging to the SAR116 clade of *Alphaproteobacteria* were depleted at the outfall. The SAR116 clade has been isolated from oligotrophic waters globally, such as the Sargasso Sea and the North Pacific Subtropical Gyre (Mullins et al. 1995, DeLong et al. 2006). While the Red Sea is naturally oligotrophic, the outfall site was characterized by nutrient enrichment. It is likely that the more eutrophic environment of the outfall was poorly suited for bacteria that thrive in oligotrophic waters, such as the SAR116 clade, but provided an ideal environment for other bacteria such as those from the class *Flavobacteria* and *Francisella* spp., as evidenced by the significant changes in sequence counts for those groups. In this way, the nutrient enrichment from the aquaculture facility appears to exert a bottom-up control on the microbial diversity near the effluent outfall.

Two limitations of this study included a lack of temporal resolution as well as site replication. These limitations prevented us from utilizing statistical approaches to compare the outfall nutrients and micro-

bial cell abundances to the other sites. We were also not able to understand the consistency of the impact to the microbial communities over time, because one seawater sample was collected directly at the outfall. In the future, we recommend sampling farther up the effluent canal, or obtaining samples from the aquaculture pools to enhance our understanding of the microbial processes occurring within the effluent canal before the effluent reaches the Red Sea.

CONCLUSIONS

In our 2009 assessment of the impact of the National Aquaculture Group facility near Al Lith, Saudi Arabia on the microbial and biogeochemical composition of the eastern Red Sea, we found that the outfall site showed elevated concentrations of ammonium and phosphate, along with changes in the microbial communities, including the presence of the potential pathogens *Francisella* spp. and *Rickettsiales*. The impacts of the effluent were more localized than hypothesized, with elevated nutrients and statistically significant microbial community alterations restricted to only the outfall site and diminishing within 500 m, likely related to the highly oligotrophic nature of these waters. Aside from this seemingly localized impact, we also noticed a shift in the microbial communities with increasing distance from the outfall across the entire study area. The impact of the aquaculture facility on the entire coral reef ecosystem was not addressed here, but our data for the waters overlying the reef near the effluent canal suggests that this ecosystem may indeed be impacted by the aquaculture effluent, and these reefs and their water quality should be monitored in the future. This study expands on the existing knowledge of the impacts aquaculture imposes on oligotrophic coral reef ecosystems, although since 2009, the increased aquaculture output in the eastern Red Sea may have altered the observed impacts. As the global aquaculture industry continues to expand to meet the demands of population growth, studies of this nature will be critical in order to assess the impacts that this industry imposes on the surrounding marine environment, especially in oligotrophic areas harboring sensitive coral reef ecosystems.

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APPENDIX

Table A1. Summary of sampling sites, sequence reads, and alpha diversity metrics. MED: minimum entropy decomposition, na: not applicable, OTU: operational taxonomic unit

Sample site	Distance to outfall (km)	No. of reads	Sub-sampled reads	No. of reads after MED	No. of OTUs	Chao1	Inverse Simpson
8	0	23149	10200	9059	291	332	17.5
7	0.5 W	27653	10200	9132	317	352	12.3
6	1.5 S	15503	10200	8853	240	262	19.3
9	1.5 N	34380	10200	9473	325	353	6.92
5	2.5 S	30509	10200	9538	295	358	5.35
12	2.5 SW	23761	10200	9161	294	353	9.23
10	3 NW	18933	10200	9369	341	375	10.7
17	4.5 SW	19276	10200	9025	351	370	25.7
11	5 NW	27524	10200	9406	338	358	6.91
19	5 N	16656	10200	9075	344	358	20.9
4	5.3 S	15755	10200	9327	292	326	6.80
25	9 S	18132	10200	9205	225	250	3.58
16	9.5 SW	23822	10200	9115	326	342	15.2
3	10 S	27600	10200	9336	332	360	18.1
18	10.1 NW	28537	10200	9035	340	369	17.1
20[a]	10.7 N	266	na	na	na	na	na
14	14.5 SW	24107	10200	9258	301	317	18.4
2	15 S	33282	10200	9000	342	357	25.9
22	15.2 NW	34679	10200	8908	337	367	14.3
13	20 SW	22209	10200	9157	310	329	31.0
23	20.3 NW	55182	10200	9032	337	361	26.1
24	20.5 S	10200	10200	9365	272	297	4.68
1	21.5 S	24170	10200	8894	353	370	16.3
21	21.7 N	45407	10200	8881	357	375	13.8

[a]If the number of sequence reads was <10200, the site was not included in microbial community analyses