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Sulphur-cycling bacteria and ciliated protozoans in a *Beggiatoaceae* mat covering organically enriched sediments beneath a salmon farm in a southern Chilean fjord

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ABSTRACT

The colourless mat covering organically enriched sediments underlying an intensive salmon farm in Estero Pichicolo, southern Chile, was surveyed by combined 454 PyroTag and conventional Sanger sequencing of 16S/18S ribosomal RNA genes for *Bacteria* and *Eukarya*. The mat was dominated by the sulphide-oxidizing bacteria (SOB) *Candidatus* Isobeggiatoa, *Candidatus* Parabeggiatoa and *Arcobacter*. By order of their abundances, sulphate-reducing bacteria (SRB) were represented by diverse deltaproteobacterial *Desulfobacteraceae*, but also within *Desulfobulbaceae*, *Desulfuromonadaceae* and *Desulfovibrionaceae*. The eukaryotic PyroTags were dominated by polychaetes, copepods and nematodes, however, ciliated protozoans were highly abundant in microscopy observations, and were represented by the genera *Condylostoma*, *Loxophyllum* and *Peritromus*. Finally, the abundant *Sulfurimonas*/*Sulfurovum* also suggest the occurrence of zero-valence sulphur oxidation, probably derived from *Beggiatoaceae* as a result of bacteriovorus infaunal activity or generated as free S^0 by the *Arcobacter* bacteria. The survey suggests an intense and complex sulphur cycle within the surface of salmon-farm impacted sediments.

1. Introduction

Farming of salmon and trout (hereafter salmonids) has increased over recent decades, with Chile and Norway accounting for nearly 70% of world-wide salmonid aquaculture production in 2012, and Chilean salmonid production reaching nearly 817,000 tons valued at US $3.81 billion (FAO, 2012). This production corresponded mostly to Atlantic salmon *Salmo salar* (48.9%), Rainbow trout *Oncorhynchus mykiss* (31.1%) and Coho salmon *Oncorhynchus kisutch* (19.8%). The aquaculture industry in Chile began in the 1980s with salmonids grown to commercial size in net-pens in the Inland sea within the Región de Los Lagos, southern Chile, to the south of the main port town of the zone (Puerto Montt) (41.4–43.3° S). Even though this activity is currently expanding southwards (as far as 46° S), it still remains strongly concentrated in the original zone, where fish-pens are mainly located within coastal areas of the shallow inner sea and shallow fjords (down to 70 m) (Holmer, 2010).

One of the main global environmental concerns with respect to fish farming operations is the local impact on the sea bed beneath the fish pens, where concentrated loads of waste food and faecal matter cause strong organic enrichment of the sediments, increasing the metabolism of the prokaryotes involved in the remineralization of this material (Macleod et al., 2004; Mente et al., 2006; Valdemarsen et al., 2012). In general, sediment bacteria representing both aerobic and anaerobic transformation pathways for the final mineralization of the organic matter are dominated by bacteria involved directly or indirectly in the cycling of sulphur (Asami et al., 2005; Hargrave et al., 2008). Under regular organic loads in coastal marine sediments, sulphate-reducing bacteria (SRB) are responsible for degrading up to 50% of all organic matter by means of a dissimilarity reduction of sulphate which leads to the production and accumulation of hydrogen sulphide in marine sediments (Jørgensen, 1982). However, under strong organic enrichment and oxygen depletion, carbon mineralization fluxes become completely driven by anaerobic bacteria that increase the accumulation and emissions of sulphide and also methane, causing a deterioration in benthic habitat quality (Hargrave et al., 2008; Holmer, 2010; Holmer et al., 2005). Under these conditions, the high rates of the dissimilarity reduction of sulphate lead to a higher production of sulphide that can reach the intertidal ($H_2S + HS^- + S^{-2}$ commonly indicated and measured as total free S^{-2}). In the upper strata of the sediment the rising S^{-2} supports the enrichment of mat forming sulphide-oxidizing bacteria (SOB) such as *Thioploca*, *Beggiatoa*, *Thiomargarita* or *Arcobacter* (Grünke et al., 2011). The most frequently observed are the large filamentous sulphur bacteria that belong to the genera *Beggiatoa* [hereafter referred to as *Beggiatoaceae* due to the taxonomic reorganization of this family and the proposal for new genera such as *Candidatus* Isobeggiatoa, *Candidatus* Parabeggiatoa and others (Salman et al., 2011)] which maintains a chemolithotrophic metabolism using oxygen or internally stored NO_3^- to oxidize sulphide to sulphate and accumulate zero-valence sulphur (S^0) intermediary as colourless granules inside the cells (Jørgensen et al., 2010; Nelson and Jannasch, 1983; Teske and Nelson,

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2006). Due to the optical properties of these granules, *Beggiatoaceae* cells mostly appear as white mats easily observed on submarine videos, the reason why they are widely considered as a good visual indicator of the biological response to organic enrichment of sediments due to aquaculture (Giles, 2008; Hamoutene, 2013, 2014; Hargrave et al., 2008; Macleod et al., 2004; Saravanakumar et al., 2012). However, even though there are plenty of studies that address the structure, physiology and taxonomy of this interesting group of bacteria in benthic marine environments where they proliferate naturally, such as submarine sulphides at cold seeps, hydrothermal vents, seasonally hypoxic marine sediments or shelf oxygen minimum zones (Dale et al., 2013; Jørgensen et al., 2010; Larkin et al., 1994; MacGregor et al., 2013; Nelson et al., 1986a,b; Powell et al., 2012; Prince et al., 1988; Salman et al., 2011, 2013; Schulz and Jørgensen, 2001), virtually no studies have been conducted to address those aspects of bacterial mats proliferating worldwide in sediments enriched by aquaculture (Saravanakumar et al., 2012). These studies could be key tools for understanding how impacted sediments respond to organic enrichment during intensive salmon culture, during the coordinated resting phase between culturing cycles, and during the recovery of the sediments after a fish farm has been removed.

Previously, we have undertaken sampling of mat-forming cells beneath a salmon farm near Calbuco Island (Aranda et al., 2010, Google Map). Gliding filamentous *Beggiatoaceae* were collected as mat forming biomasses that allowed for the construction of bacterial clone libraries where *Beggiatoaceae* was represented. Additionally, *Deltaproteobacteria*, *Sulfurospirillum* sp., *Sulfurovum* sp. and *Fusibacter* sp. were detected, suggesting the occurrence of an accelerated and complex sulphur cycle in the upper most layer of the organically enriched sediment. However, the main limitation of that work was the low number of clones available for phylogenetic analysis which prevented establishing with certainty the relative composition of the bacteria inhabiting the mat. Additionally, ciliated protozoans dominated the benthic infauna in the collected biomass (not reported), but no effort was made to identify the ciliate or ciliates involved. Unfortunately, we could not continue the study due to a lack of permission for accessing the fish farm.

Recently, we had access to a colourless *Beggiatoaceae* mat beneath a relatively small salmonid farm in Estero Pichicolo (Fjord). Taking advantage of this opportunity the aims of this research were: (1) to assess *Bacteria* and *Eukarya* community composition in the mat by using 454 PyroTag as a massive parallel high-throughput sequencing technique, (2) to compare annotations occurring in both mat-forming bacterial biomass collected directly and after cleaning in order to determine if the accompanying bacteria are attached to the mat-forming cells, or are just inhabiting the mat or the upper most layer of the sediment, and (3) to conduct conventional Sanger sequencing to obtain more precise taxonomic information on the mat forming and accompanying bacteria.

2. Methods

2.1. Study site

Estero Pichicolo is a small fjord located in the Región de Los Lagos. As with most of the fjords in the zone, it supports diverse artisanal benthic fisheries coexisting with extensive and intensive aquaculture operations (mainly mussel and salmon respectively). The study site was a Rainbow trout farm with a net production close to 600 tons per culture cycle (10–12 months), which is low compared to other salmonid farms in the region that produce more than 3000 tons per year. Preliminary submarine image inspections at this site revealed an almost complete covering of sea bed underneath the farm by a white *Beggiatoaceae* mat, which was absent beyond the influence of the farm according to the dominant local marine current, in a radius that ranged between 20 and 60 m. The sampling point was the seabed underneath the perimeter of the farm (42°2′13″S, 72°35′33″W, Google Map). A reference site 270 m downstream with no visible microbial mats was also chosen for preliminary analyses. Water depth at the study and reference sites was 35 to 43 m depending on the state of the tide.

2.2. Sample collection and preliminary analysis

Samples were collected on January 31, 2012, two weeks after the complete fish harvesting at the end of one farming cycle. Sea bed sediment mini-core samples (24 mm diameter) were collected by scuba diving using polypropylene 50 ml conical centrifuge tubes with the bottoms cut off and sealed with a rubber bung after sampling. pH and redox potential (Eh) were measured in the field by conventional electrodes to a depth of 2 cm (pH meter 330i, WTW, Weilheim, Germany). Samples were transported on ice in the dark for processing within 2 h of collection. For preliminary analyses, 3 mini-cores per site were sliced at 1-cm intervals (0 to 3 cm depth) and stored in plastic bags, frozen at −80 °C and freeze-dried before being transferred to a muffle furnace for 4 h at 450 °C. The weight of organic material was calculated as the difference between freeze-dried and final furnace ashed weights. Additionally, 9 mini-cores per site were selected for minimal disturbance and the presence of 9 to 14 ml of overlying-sea water, which was gently extracted and replaced with sterile oxic sea water (SOSW, made by a combination of degassed and air-saturated 0.2 mm filter sterilized sea water to achieve 8.1 to 9.2 mg O_2/l). After water replacement, these samples were incubated at 10 °C in the dark to allow oxygen consumption and gliding of mat forming cells in the overlaying-sea water (Kojima and Fukui, 2003). Gliding filamentous mat-forming cells were gently aspirated with a disposable Pasteur pipette, and then transferred to 100 ml polypropylene flasks filled with SOSW. Filaments were gently dispersed by pipetting followed by flushing with SOSW. Samples were then incubated for 3 h at 4 °C to allow for the decantation of filaments and chemotactic self-aggregation. The mat-forming filament cells exhibit a self-aggregating behaviour forming a dense white mass of motile filaments at the centre of the base of the container. Combined mat biomasses of the study site were segregated into two subsamples for independent downstream processing, one with some barely visible granules of sediment was downstream processed directly (dirty mat biomass) and the rest was cleaned in order to reduce the carryover of sediment particles, flora not attached to the gliding filamentous cells and the observed polychaetes and oligochaetes that could saturate the *Eukarya* library. Cleaning was performed three times by dispersion in fresh SOSW followed by cell self-aggregations steps. Dirty and clean mat biomasses were then examined with an inverted light microscope with attached digital camera (Olympus CKX41). Non-mat biomass was collected from the reference site. Total DNA was extracted from the dirty and clean mat biomasses using a Power Soil DNA isolation Kit (MO BIO Laboratories, Inc., CA) according to the manufacturer's specifications.

2.3. Sanger sequencing

Genomic DNA of dirty mat biomass was PCR amplified using primers targeting the almost complete 16S rRNA gene of *Bacteria* (primers 8f-AGAGTTTGATCCTGGCTCAG and 1492r-CGG TTA CCT TGT TAC GAC TT). The 50-μl high-fidelity-PCR mixture contained 10 μl of the primer set (10 μM each), 0.5 μl (2.5 U/μl) of Pfu DNA Polymerase (recombinant) (Thermo Scientific), 5 μl of 10× Pfu buffer with 20 mM $MgSO_4$, 0.5 μl of deoxyribonucleotide triphosphate mixture (2 mM each) and 50 ng of DNA template. The PCR programme contained a hot start and an initial denature at 94 °C for 5 min, followed by 25 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min. The PCR product was purified using Axy Prep PCR Cleanup Kit (Axygen), poly-A-3′ extension was made with Taq Polymerase (Invitrogen), cloning was performed with a TOPO TA Cloning Kit (Invitrogen) using *Escherichia coli* JM107 made competent with a Transform Aid Bacterial Transformation Kit (Thermo Scientific), all steps following the manufacturers instructions.

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