Substrate composition and relationship to bacterial communities during production and fallow periods at hard-bottom aquaculture sites in Newfoundland

by

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Abstract

Newfoundland salmonid aquaculture is established in deep, sheltered bays and above hard-bottom substrates. Over time, organic waste accumulates on the seafloor, changing sediment chemistry and community structure. Visual markers including white bacterial mats are used to monitor organic enrichment but alternative methods may be more informative. I sampled substrates at two aquaculture sites in Newfoundland [experiencing production (active) and fallow (inactive) conditions], characterized abiotic features and bacterial communities, and tested for associations with bacterial mats. Organic enrichment and abiotic markers of aquaculture were prominent during both production and fallow periods, especially near cages. Bacterial mats were associated with elevated concentrations of organic matter, Zn, Cd and Cu. Bacterial community clusters show relationships with organic matter and Zn, and mats were associated with all identified bacterial community clusters. This research highlights the potential use of bacterial and abiotic markers for monitoring organic enrichment and site recovery at aquaculture sites in Newfoundland.

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List of Abbreviations and Symbols

B.C. – British Columbia

°C – Degree Celsius

C – Carbon

Ca – Calcium

Cd – Cadmium

CHN – Carbon/Hydrogen/Nitrogen

Co – Cobalt

Cr – Chromium

Cu – Copper

DFO – Department of Fisheries and Oceans

Fe – Iron

h – hour

HCL Acid – Hydrochloric Acid

HNO_3 – Nitric Acid

H_2O_2 – Hydrogen Peroxide

m – metres

mg – milligram

Mn – Manganese

N/A – Not Applicable

N – Nitrogen

Ni – Nickel

N.B. – New Brunswick

N.L. – Newfoundland and Labrador

NLDFA – Newfoundland and Labrador Department of Fisheries and Aquaculture

N.S. – Nova Scotia

NWAFC – Northwest Atlantic Fisheries Centre

OE – Organic Enrichment

OM – Organic Matter

OPCs – Opportunistic Polychaete Complexes

P – Phosphorous

Pb – Lead

ppm – parts per million

Se – Selenium

SOM – Sedimented Organic Material

TOC/TN Ratio – Total Organic Carbon/Total Nitrogen Ratio

Zn – Zinc

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Chapter 1. Introduction and Overview

1.1 Global aquaculture market

Aquaculture is a key industry that provides food (aquatic fauna and flora), employment and economic opportunities to many developed and developing countries worldwide. Two hundred and two countries and territories contribute to the current aquaculture industry that has been on a marked rise in growth in the past few years, with an average annual production growth rate of 5.8% for 2001-2016 (FAO, 2018, 2019). As wild fisheries production remains static, aquaculture alleviates the demand for fish as the global population increases; aquaculture contributed 47% of global fish product in 2016 (FAO, 2018). Currently, Asian countries dominate the global market with China, India and Indonesia as the top three aquaculture producers globally (FAO, 2019). There are currently 608 species farmed in aquaculture, with product composition differing by region (FAO, 2019).

In Canada, finfish comprises the largest portion of the aquaculture market with 151,342 tonnes produced in 2017 (DFO, 2018), contributing to the 53.4 million tonnes produced globally within the same year (FAO, 2019). Canadian aquaculture production has increased substantially since the introduction of salmonid farming in the 1970s and Canada is now considered the 4th largest salmon producer globally (DFO, 2013). Provinces contributing to salmonid production are British Columbia (BC), New Brunswick (NB), Nova Scotia (NS) and Newfoundland and Labrador (NL), with Atlantic salmon (*Salmo salar)*

production being well established in all four provinces (Manning and Hubley, 2016). Canadian aquaculture is managed through different levels of government: federal, provincial and territorial, and is regulated under several acts, legislations and regulations. The management of local aquaculture sites is overseen by provinces, or federally for BC, with several regulatory regimes in place depending on geographical location (FAO, 2017; DFO, 2019).

1.2 Newfoundland aquaculture

Nationally, NL is the 3rd largest producer of salmon (Manning and Hubley, 2016). The NL aquaculture industry has expanded quickly since implementation in the 1980s and has a positive impact on rural communities by providing employment and economic opportunities (Anderson et al., 2005; NLDFA, 2014). Most finfish aquaculture is established along the Southern coast of NL in the Bay D'Espoir region (Fig. 1.1) where the coastline is characterized by sheltered deep bays (>30 m) and fjords (Anderson et al., 2005; DFO, 2014). In 2015, 87 salmonid aquaculture licences were active, with sites occupying a total water area of approximately 2,402 hectares (NLDFA, 2019). In the same year, NL produced 19,684 tonnes of salmonid product, equaling $148.5 million in value (NLDFA, 2019). Sites typically hold between 250,000- 1,000,000 kg of biomass depending on how many cages are present. As an initiative to expand salmonid aquaculture in NL, the government approved a $250 million aquaculture project in Placentia Bay that is expected to be fully operational by 2025 (NLDFLR, 2018).

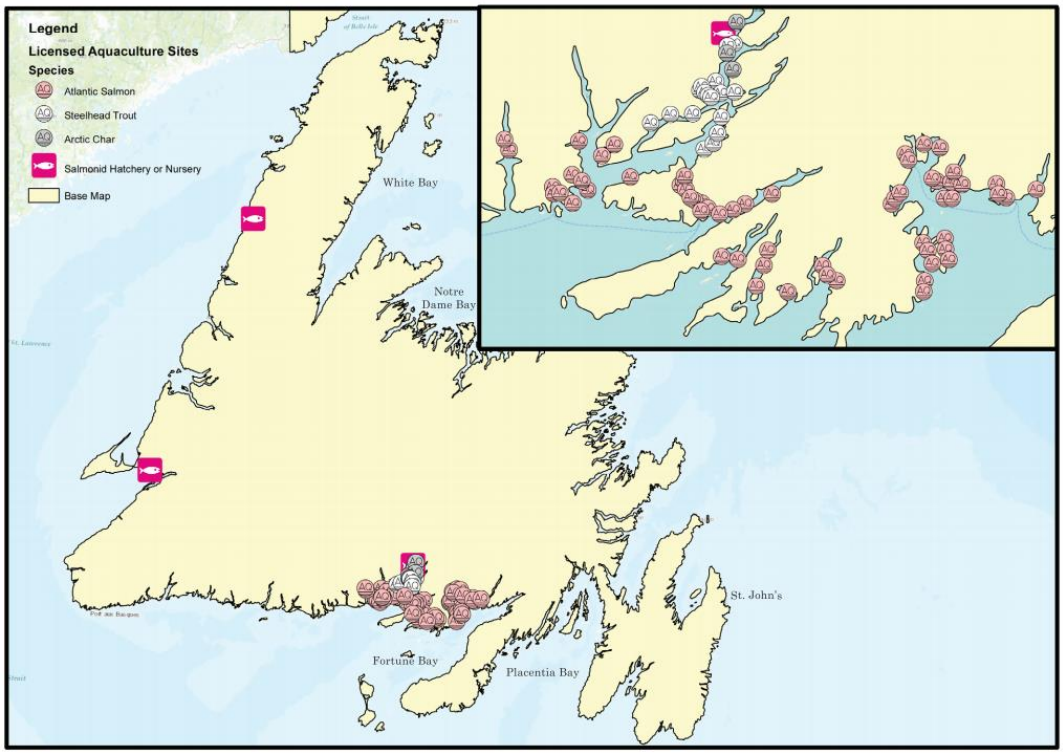

Figure 1.1. Licenced salmonid aquaculture sites in Newfoundland in 2013. Source: NLDFA (2014).

NL licenced sites can either be active ("in production") where salmon cages are stocked and harvested within a 2-year period or inactive ("in fallow") where production cycles are paused and cages are removed. In accordance with the NL Aquaculture Act, fallowing periods are required between production cycles to mitigate the spread of pathogens/disease and environmental degradation at finfish sites (NL Aquaculture Act, 2013). Fallow duration for Atlantic salmon- producing sites depends on the minimum period set by Bay Management Area (BMA) principles which are a minimum of 7 months for sites and 4 months for a BMA. (NLDFLR, 2019a). Salmon die-off caused by disease or other environmental factors can also result in longer fallowing durations (NLDFLR, 2019a).

1.3 Benthic environmental effects

While NL aquaculture has made a positive socioeconomic impact in the province, there has been an increase in public and scientific concern over potential negative effects on the local environment. Common environmental concerns associated with finfish aquaculture include: fish escapements, disease transfer (ex. infectious salmon anemia) and pollution (Nogales et al., 2011). Pollutants originate from the use of antibiotics, antifoulants, parasiticides as well as organic waste (Cabello, 2006; Guardiola et al., 2012; Hamoutene et al., 2018).

Organic waste deposition at farm sites and its potentially serious effects on the benthos have been widely studied around the world including: Newfoundland (Anderson et al., 2005; Hamoutene et al., 2016; Verhoeven et al., 2016; Salvo et al., 2017a), other parts of Canada (Brooks et al., 2003; Chou et al., 2004), Europe (Carroll et al., 2003; Borja et al., 2009), Oceania (Keeley et al., 2015), West Asia (Agah et al., 2013) and East Asia (Yokoyama et al., 2006). Organic sediment enrichment originates from the discharge of uneaten fish feed pellets, fish faeces and other organic inputs that settle onto the seafloor (Fig. 1.2) (Olsen et al., 2008; Wang et al., 2012). Deposits of this enriched sediment, commonly known as 'flocculent matter', may accumulate over time and can lead to hypoxic and anoxic conditions as organic matter undergoes continued bacterial degradation and increased benthic oxygen demand (Panikov, 1995, Hargrave et al., 2008), altering sediment chemistry (Shaanning, 1994; Hamoutene, 2014) and natural benthic communities (Pearson and Rosenberg, 1978). Studies have shown that flocculent matter deposits cause decreases in natural species diversity and lead to dominance by opportunistic/tolerant taxa (Brooks et al., 2003; Zhulay et al., 2015). Macrofaunal taxa that are most sensitive to changes

associated with oxygen depletion include malacostracan crustaceans, bivalves and gastropods (Diaz and Rosenberg, 1995). Trace elements have also been known to settle on the seafloor underneath cages from sources such as fish feed and antifoulants (Dean et al., 2007; Thomas and Brooks, 2010), with copper being the most frequently used biocide (Guardiola et al., 2012).

As particulate organic matter (and associated trace elements) disperse along a spatial gradient, concentrations typically decrease as distance from cages increases (Ye et al., 1991; Carroll et al., 2003). The effects of organic enrichment to the benthos have been noted at 300 m from cages (Yokoyama et al., 2006) but are most apparent at distances of <50 m (DFO, 2003; Kalantzi et al., 2013). Macrofaunal assemblages have also been shown to become disturbed close to cages and diverge along gradients as they react to changes in sediment chemistry (Karakassis et al., 2000; Cathalot et al., 2012). The rate of deposition at an active farm site is dependent on parameters of the farm and the hydrodynamics of the site, with the latter also influencing spatial patterns (Sanz-Lázaro and Marín, 2011).

Temporal trends have also been investigated to understand how organic enrichment progresses over time and the nuances of benthic remediation during fallowing periods. Studies from different regions around the world indicate that chemical and biological recovery times can vary from a few months (Brooks et al., 2003) to years (Karakassis et al., 1999; Brooks et al., 2004; Keeley et al., 2014); factors such as depth, topography, natural sediment regime (i.e. soft-bottom vs. hard-bottom sites) and production cycle parameters can influence recovery rates. Verhoeven et al. (2018) reported little to no recovery in microbial communities at a hard-bottom site in Newfoundland even after >35 months in fallow. Chemical conditions of sediment notably recover faster than macrofaunal

communities (Macloed et al., 2004) where areas closest to cages will likely experience reduced conditions longer than areas furthest away (Pereira et al., 2004).

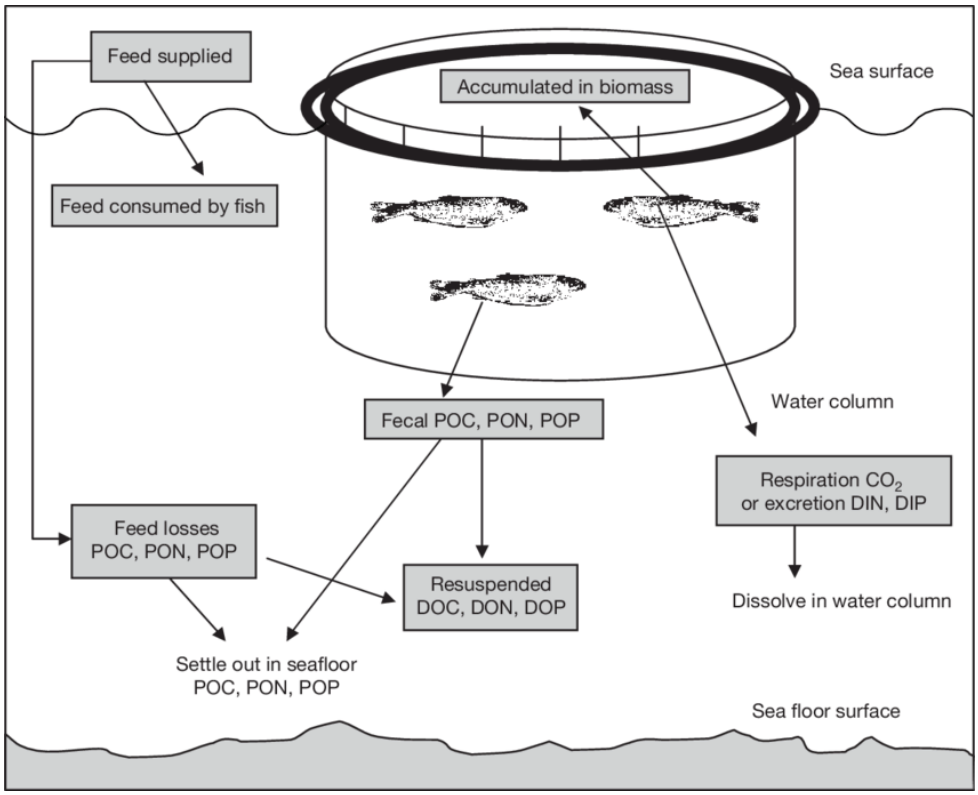

Figure 1.2. The flow and fate of organic waste from salmon cages during production. Particulate organic carbon, nitrogen and phosphorus (POC, PON and POP, respectively) originate from fish feed and fish feces and settle onto the seafloor. Source: Wang et al. (2012).

1.4 Monitoring for organic enrichment

Compared to other provinces, NL has unique features in terms of biogeography, oceanography, climate and marine resources; features of particular importance to aquaculture and its regulation include predominantly hard-bottom substrates and weak near-bottom currents, temperatures and dissolved oxygen levels at the seafloor (AMEC, 2002; Manning and Hubley, 2016). Licensing salmon farms in NL requires sites to be deeper than 30 m to provide enhanced waste dispersal (NLDFLR, 2019b). According to Canadian regulations, aquaculture sites must undergo routine monitoring of benthic organic enrichment, with common methods including measuring free sulfide and redox potential in sediments collected via grab sampling at sites. However, as NL is dominated by hard-bottom substrates, obtaining enough sediment for sulfide and redox determination is difficult (Hamoutene et al., 2013). In fact, a recent study stated that the success rate of usable grabs (>5 cm of sediment) performed on the South Coast of NL was only 44.5% (DFO, 2014). As an alternative, drop-camera monitoring is currently being utilized at NL sites in accordance with DFO monitoring practices (DFO, 2012, 2015) and regulation (AAR, 2018). Images extracted from videos provide a permanent 'snapshot' of the condition of the seafloor as well as a record of the fauna and flora present. More importantly, video data also allows for the identification of common biotic indicators of organic enrichment: white mat-forming, sulfate oxidizing bacteria (Fig. 1.3) and Opportunistic Polychaete Complexes (OPC) (Fig. 1.4), both of which are highly associated with flocculent matter deposition at NL farm sites (Hamoutene et al., 2013; Hamoutene, 2014) (Fig. 1.5). These biotic indicators may thrive under a range of organic enrichment

conditions but may be absent beyond certain environmental thresholds, and cannot be used to categorize intermediate degrees of enrichment (Salvo et al., 2017a,b, 2018).

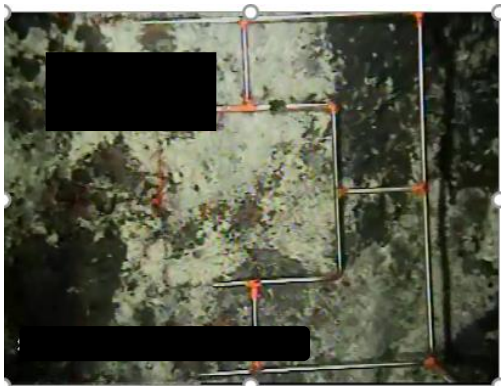

Figure 1.3. Example of white bacterial mats on the surface of sediment buildup (flocculent matter) at a site in fallow in NL.

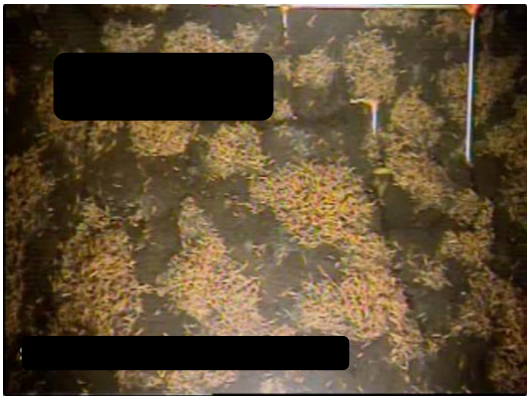

Figure 1.4. Example of OPC clusters on the surface of sediment buildup (flocculent matter) at a site in production in NL.

Figure 1.5. Example of white bacterial mats and OPC clusters on the surface of sediment buildup (flocculent matter) at a site in production in NL.

Advances in gene sequencing technology have allowed researchers to explore alternative ways to monitor seafloor conditions at aquaculture sites by characterizing bacterial communities. Bacteria make good candidates for measuring organic enrichment at aquaculture sites because they respond rapidly to inputs in organic matter (Panikov, 1995) and are sensitive to changes in sediment quality (Nogales et al., 2011; Dowle et al., 2015; Wan et al., 2017). Studies have already investigated the relationships between bacterial community composition and organically enriched sediment at aquaculture sites in the Bay D'Espoir region in NL (Verhoeven et al., 2016, 2018). In particular, bacterial communities were found to be useful for characterizing intermediate levels or stages of organic enrichment in NL (Verhoeven et al., 2018).

1.5 Thesis objectives

With the rapid expansion of aquaculture in the province, it is increasingly important to properly monitor the organic enrichment taking place underneath cages at salmon

producing sites. However, there is still much to learn about organic waste that has settled on the seafloor during production and fallowing periods in both time and space in this region. To understand the dynamics of organic waste deposition on hard-bottom substrates at NL aquaculture sites we must first characterize the abiotic features and bacterial communities characterizing the sediment on spatial and temporal scales. We must also link abiotic features and bacterial community composition of flocculent matter to visual biotic indicator data obtained via video surveys to either strengthen current monitoring methods or justify the need for implementing new monitoring tools (e.g. sequencing-based approaches) at aquaculture sites.

The second chapter of this thesis aims to describe abiotic characteristics of flocculent matter at NL aquaculture sites and associate those characteristics with bacterial mat presence/absence data. Specifically, sediment and/or flocculent matter samples were collected across transects (i.e. sampled underneath cages and at increasing distance intervals) and at different times (i.e. sampled twice within a two year period) from a production and a fallow site in the Bay D'Espoir region, and concentrations of organic matter and trace elements were determined from those samples. Relationships between bacterial mat presence and abiotic characteristics were examined and spatio-temporal trends in abiotic features explored.

The third chapter relates bacterial community composition in sediments experiencing organic enrichment (production) or remediation (fallowing) to sediment abiotic characteristics and bacterial mat data analyzed in chapter 2. Bacterial communities were categorized based on the biotic indicator community analysis performed in (Verhoeven et al., 2018) via high-throughput sequencing of the 16s rRNA gene in DNA.

Lastly, the fourth chapter summarizes findings from the research chapters and suggests future work to advance research and monitoring practices in this field.

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Chapter 2. Characterization of flocculent matter at Newfoundland aquaculture sites in production and in fallow

2.1 Introduction

2.1.1 Organic enrichment at salmonid aquaculture sites

Salmon aquaculture plays an important role in the global food market as the demand for salmon and other seafood products increases (FAO, 2016). In 2016, 54.1 million tonnes of finfish (including salmon) were produced through aquaculture, accounting for more than half of the 80 million tonnes of aquaculture products yielded globally that year. Atlantic Salmon (*Salmo salar*) is one of the most produced species worldwide in terms of volume (FAO, 2018).

Canada is ranked 4th in salmon production globally, with Norway, Chile and Scotland leading as the top three producers (Manning and Hubley, 2016). Canada's success is in part due to its extensive coastline and ideal conditions for salmon farming, with British Columbia accounting for most of the aquaculture output in terms of value (Nguyen and Williams, 2013). Newfoundland (NL) is also an important salmon producer; this activity brings many employment and economic opportunities to coastal communities, especially along the South coast. Total NL finfish aquaculture production in 2018 was 15,107 tonnes and was valued at $134 million (Statistics Canada, 2020).

With the rapid global expansion of salmon aquaculture, there are growing concerns over potentially negative impacts to the marine environment. Salmon production consists in

stocking and growing fish in high densities within suspended pens along the coastline. These production cycles typically last between 1–2 years (DFO, 2014). Salmon production requires feeding the fish using large numbers of fish pellets. Over time and through several production cycles, excess organic waste is generated and ultimately enters the ocean ecosystem. Waste material can settle on the seafloor and form flocculent matter, defined here as a mixture of components that originate mostly from uneaten fish food pellets and fish feces (Strain and Hargrave, 2005; Salvo et al., 2015). Material that does not settle to the seafloor near the farm site is suspended into the water column and dispersed farther away via ocean currents (Wang et al., 2012). Wastes that settle on the seafloor may also experience subsequent resuspension and transport through bottom currents (Environment Canada, 2009).

Fish are fed dry, nutrient dense pellets that are formulated to meet the dietary needs of the fish and to promote overall health and growth until harvest (Manning and Hubley, 2016). Although fish pellets vary in composition along with fish growth and period of the year, fish pellets are typically composed of 50% carbon (C), 6% Nitrogen (N) and 1% Phosphorus (P) (Olsen et al., 2008). Fish pellets that are uneaten can settle to the seafloor underneath aquaculture sites, while digested fish pellets enter the system through defecation (Wang et al., 2012). Some of the particulate organic C, N and P may remain suspended in the water column (Troell et al., 2009). Fish feces likely make a greater contribution to flocculent matter than uneaten pellets (Wang et al., 2013); salmon feces can have a notably high P content (4%; Kristiansen and Hessen, 1992).

Over time, sedimented organic material (SOM) that accumulates at fish farm sites is partially decomposed by bacteria, resulting in changes to the natural benthic environment

(Carroll et al., 2003; Strain and Hargrave, 2005; Olsen et al., 2008); as more flocculent matter accumulates, oxygen consumption by bacteria exceeds supply and consequently lowers oxygen availability within the sediment. This can lead to hypoxic and anoxic conditions along with elevated concentrations of sulfides and/or methane, the latter being produced by anaerobic microbes (Hargrave et al., 2008).

Other compounds found at the seafloor underneath aquaculture sites can originate from biofouling paints used on fish cages to prevent settlement and adhesion of organisms (Thomas and Brooks, 2010). Copper (Cu) and Zinc (Zn) are basic components of biofouling paint that can leach into the water column and eventually settle to the bottom. In fact, Cu and Zn have been found in higher concentrations at farms using the paint (Nikolaou et al., 2014) and tend to show higher concentrations near cages (Sutherland et al., 2007; Russell et al., 2011); however, the industry seems to be evolving towards using mechanical net washers for mitigating biofouling. Zn, Cd and Cu are also components of fish diets (Dean et al., 2007) and Zn and Cd were found in high abundance in farmed fish manure, along with manganese (Mn), Chromium (Cr), Lead (Pb) and Iron (Fe) (Naylor et al., 1999). Cu and Zn concentrations (and organic carbon) have also been shown to increase as oxygen levels in sediment decrease under salmon cages (Chou et al., 2004).

2.1.2 Biotic indicators of aquaculture waste

Intensive salmon farming can produce drastic changes to the benthic environment from organic loading, making it important to monitor these effects. Most monitoring at soft-bottom aquaculture sites is completed by investigating redox potential, sediment sulfides and/or changes in benthic community composition from sediment obtained using grabs (Carroll et al., 2003). In fact, Canadian Aquaculture regulations state that potential benthic impacts are normally monitored by measuring sediment sulphide (DFO, 2012). However, collecting sediment samples using grabs at NL aquaculture sites is challenging given that substrates in this region are mainly rocky (Hamoutene, 2014; Hamoutene et al., 2015).

As an alternative to common Canadian monitoring practices, drop-camera visualization is currently being used in NL to monitor organic enrichment by documenting the presence of two biotic indicators: white bacterial mats and opportunistic polychaete complexes (OPCs) (Hamoutene et al., 2015), as well as barren substrates where organisms were recorded prior to aquaculture production but are no longer observed (AAR, 2018). Reduced sediment conditions may develop during production and allow white bacterial mats and OPCs to thrive; however, the range of environmental conditions present at aquaculture sites can exceed the tolerance ranges of these organisms, and therefore they are, by themselves, imperfect indicators of aquaculture activity (Salvo et al., 2017a,b, 2018a). In NL, little is known on the relationship between biotic indicators and organic enrichment during the full extent of production and fallow periods and under different environmental conditions, though it was found that bacterial mats develop over a broader range of depths and temperatures than OPC (Hamoutene et al., 2016, Salvo et al., 2017a). More research is needed to learn how and why these indicators grow under reduced sediment conditions. In

this study, we explore trends associated with bacterial mats, which are the more commonly encountered biotic indicator of aquaculture in NL sites (Hamoutene et al., 2016), and do not consider OPCs.

White bacterial mats found at NL aquaculture sites were originally assumed to be *Beggiatoa* sp., but Verhoeven et al. (2016) showed, via 16S rRNA gene sequencing, that mats at cage edge were dominated by *Spirochaeta* (15%), *Prevotella* (21%), *Meniscus* (11%) and *Odoribacter* (20%). At NL aquaculture sites, mats are found most frequently within 10 m from cages but are commonly observed up to 100 m away; mats are visible during production and can persist into fallowing periods (Hamoutene et al., 2016; Salvo et al., 2017a). Bacterial mats have been observed on all types of substrates and are strongly associated with flocculent matter deposition via aquaculture activity in NL (Hamoutene et al., 2013, 2015, 2016; Salvo et al., 2017a). Although sediment sulfide and redox values differ significantly at stations with and without bacterial mats (Hamoutene, 2014), there has been no work investigating relationships between bacterial mat presence and a broader range of abiotic sedimentary factors (e.g. OM content, elemental markers of aquaculture) at different distances from cage during periods of production and fallowing in this region.

2.1.3 Spatial and temporal changes in sediment enrichment linked to aquaculture

Considerable research has been done to investigate spatial and temporal changes in SOM content at aquaculture sites. Many studies have shown that organic waste content is high near cages (Karakassis et al., 2000; Sarà et al., 2004; Corner et al., 2006), and that effects of organic enrichment can decrease along a spatial gradient (Ye et al., 1991; Carroll et al., 2003) as far as 300 m from cages (Yokoyama et al., 2006). Fewer studies have

examined temporal changes in SOM at aquaculture sites during production. Many factors such as farming intensity, farm location and age, bathymetry and currents can influence temporal shifts in benthic organic loading. Neofitou et al. (2010) showed no evidence of a significant temporal effect in organic matter and organic carbon from over a 9-month production period at a fish farm in the Mediterranean Sea. In contrast, in New Zealand, a site showed signs of recovery after 9 months of fallowing but was still impacted after 24 months (Keeley et al., 2015). Organic enrichment theoretically decreases over time as sites undergo fallowing, but this rate varies depending on location and can span from a few months to >5 years for a full recovery (Keeley et al., 2014).

2.1.4 Objectives of this study

In this study, we explore trends at NL aquaculture sites by first characterizing abiotic features of sediments (comprised of variable proportions of natural sediments and flocculent matter) sampled from NL aquaculture sites during organic enrichment (production) and remediation periods (fallowing). From these sediment samples, we quantified a series of elements recently treated as abiotic markers of aquaculture (Hamoutene et al., 2018): Phosphorus (P), Calcium (Ca), Iron (Fe), Cobalt (Co), Nickel (Ni), Zinc (Zn), Selenium (Se), Cadmium (Cd), Lead (Pb), Chromium (Cr) and Copper (Cu), as well as total organic carbon (%TOC), total nitrogen (%TN), TOC/TN and the percentage of organic matter (%OM). We examined relationships between the presence of bacterial mats and the concentration of some abiotic sediment characteristics to improve our understanding of the conditions under which these mats can be observed during routine

monitoring. Both spatial and temporal trends were also examined where feasible. Specifically, we investigated:

1) Relationships between abiotic sediment characteristics and the presence of bacterial mats;
2) Changes in abiotic and biotic sediment characteristics with distance to cage, during both production and fallow periods; and
3) Changes in abiotic sediment characteristics over two years at a site undergoing fallowing, to track potential remediation.

We hypothesize that: 1) biotic (bacterial mats) and abiotic markers (flocculent matter components) of aquaculture will show a similar pattern of decrease with increasing distance from cage; 2) bacterial mats will be positively associated with abiotic markers; and 3) concentrations of biotic and abiotic markers will decrease over the fallowing period.

2.2 Materials and methods

2.2.1 Study sites

This study was conducted in 2016 and 2017 along the south coast of Newfoundland, in the Coast of Bays region where salmonid aquaculture is prominent. Two sites were selected: a production site located in Hermitage Bay and a fallow site in Belle Bay. The exact locations of both sites cannot be disclosed at the request of industry. Samples were also collected from an additional location and treated as a reference (i.e. showing no influence from aquaculture deposition). This reference site, discovered during fieldwork in

2017 in Belle Bay, was chosen as it had natural sediment deposition and was located away from aquaculture activity (> 500 m).

The seafloor at both the production and fallow sites is dominated by bedrock, with pockets of sedimented material. At the former site, a first production cycle ended in June 2015; this was followed by an 11-month fallow period before the start of the next production cycle in May 2016, when cages were stocked with 620,000 of fish. The fallow site was first stocked in 2005 and went through a series of production cycles up to its last harvest in 2014 with a yield of 57,000 fish. The site then transitioned into a fallow period in August 2014, at which point the cages were moved to another location in the area.

2.2.2 Data collection

Sampling (video collection followed by grabs) was performed along transects running in different directions from the cage array, at stations located at 0, 20, 40, 80, 120 and 160 m from cage edge and at two additional 'center cage' stations, located in the center of a set of four cages at the production site only (Appendix A). Where cages were no longer present (fallow site), cage GPS coordinates from the last production cycle were used as a guide for determining transect placement. Sampling at the reference site (June 2017, distance from aquaculture activities > 500 m) took place at depths corresponding to those found along production and fallow transects. Sampling at the production site was performed in October 2016 (5 months into production) and in June 2017 (13 months into production). At the fallow site, sampling occurred in September 2016 (24 months into fallow) and June 2017 (34 months into fallow). It should be noted that cages at the production site were not in the

exact GPS locations and were moved a few meters from the previous sampling year due to a change in the company's feeding setup.

2.2.2.1 Video monitoring

To identify areas with a sufficient buildup of sediment or flocculent matter (where grab samples could be obtained), underwater video monitoring was performed in 2016 and 2017, in accordance with NL regulatory practices (DFO, 2012; Mabrouk et al., 2014) along transects.

Video capture was conducted using an underwater camera system (VRM-1, JW Fishers) fixed, along with lamps, to an aluminum frame with an attached 50 x 50 cm quadrate. The camera is linked via a cable to a digital video recorder and monitoring system, with light control and GPS reading. At each station, video recording started once the underwater camera system was deployed; it was then slowly lowered until it reached the bottom. To obtain a broader representation of the seafloor area at a station, this process was performed at least three more times by lifting the frame approximately 30 cm from the bottom and allowing it to drift a few feet where it was dropped down again. Recordings lasted approximately 2–5 min (per station) depending on the depth of the location, while the boat remained stationary. For each video recording, the following was noted: site name, station number, GPS coordinates, sampling date, time of day and depth (m). The presence or absence of bacterial mats and of sediment or flocculent matter within the quadrat were also documented when reviewing the footage using Image Grab software. Flocculent matter was considered present if the bottom of the aluminum cage cut through sediment that was fluffy and semi-gelatinous in appearance. Depending on the level of accumulation, the cage

penetrated within a few cm(s), and displayed important resuspension in overlying water (Salvo et al., 2018b). See Appendix B for drop-camera images illustrating the difference between the presence and absence of flocculent matter.

2.2.2.2 Grab sampling

Stations where video monitoring revealed visible soft substrates (i.e., sediment or flocculent matter) were targeted for grab sampling (see Table 2.1 for full summary of stations used in this study) using an Ekman Grab Sampler measuring 15 x 15 x 25 cm. The grab sampler was lowered down while the boat remained stationary. Once it reached the bottom, closure of the bottom shovels was triggered using a weighted messenger attached to the cable, and the sampler was brought to the surface. Station depth was determined by the on-board sounder. 9

At each preselected station along the transect, up to five attempts to obtain a successful grab sample were made before the station was abandoned. Grabs containing less than 2 cm of material were considered unsuccessful. Grab contents were photographed for visual documentation upon retrieval, and were then dumped into a bucket. Grab contents were transferred haphazardly using stainless steel spatulas into sterile two 20 mL scintillation vials for elemental analysis and one 50 mL plastic centrifuge tube for %OM and CHN analysis. Samples were placed on ice while on board and then moved to a -20°C freezer; they remained frozen until transported to the Northwest Atlantic Fisheries Centre (NWAFC), Fisheries and Oceans Canada.

Table 2.1. Summary of successful grab sampling stations along transects at production and fallow sites at each sampling year.

	2016 **Transects** (Stations)	**2017** **Transects** (Stations)
Fallow site	**8W** (0, 40, 80 m) **3S** (0, 0, 20, 40, 80, 120, 160 m) **4W** (0 m)	**8W** (0, 20, 40, 80 m) **3S** (0, 20, 40, 40, 80, 120, 160 m) **4W** (0 m) **10S** (120 m)
Production site	**4W** (0, 20 m) **4S** (0, 20 m) **1N** (*5 m) ****X2** (0 m)	**4W** (0 m) **4S** (40 m) **1N** (0, 0, 20, 40, 40 m) ****Center** (0, 0 m)

Note: reference stations (where two grabs were obtained at same GPS location) are not shown. *station was not 0 m from cage edge due to presence of gear on the edge of the cage. **station is between a set of four cages. Average depth of successful production stations = 80 ± 17 m and successful fallow stations = 80 ± 5.6 m.

2.2.3 Analytical procedures

2.2.3.1 Sample processing

All sediment/flocculent matter was processed before analysis. First, frozen samples were brought to a biogeochemistry lab facility (Memorial University, Canada) for freeze-drying. Afterwards, samples were transported to the NWAFC where fish scales, shells and debris were removed as best as possible. Samples were then manually ground and homogenized using a mortar and pestle. Processed samples were stored in a -20°C freezer to keep moisture from entering the freeze-dried sediment until needed.

2.2.3.2 Organic matter determination

The percentage of organic matter (%OM) was determined using the loss on ignition method. Subsamples of dried, homogenized samples from production, fallow and reference stations were transferred into an aluminum weighing dish and weighed (0.0001 g) on a scale (TR-204, Denver Instrument Company). Subsample weight was typically ~1 g but was as low as ~0.5 g if there was only a small amount of sediment collected from that station. For the 2016 grabs, enough sediment was obtained to produce two replicates per station, but only one replicate was prepared for 2017 grabs as there was less sediment available. Once subsamples were weighed, they were placed into a muffle furnace for 4h at 450°C to burn off organic material, leaving only the mineral component. Afterwards, %OM was calculated for each sample based on the loss in dry weight.

2.2.3.3 CHN

Elemental analyses of N and C (and H) were performed for all sediment samples. Subsamples of dried, homogenized sediment were retained to measure the percentages of total nitrogen (%TN) and total organic carbon (%TOC) from each station. Sediment weights used for %TN and %TOC analysis were based on %OM: we used 3-4 mg of dried sediment where %OM ≥ 40% and 6-10 mg where %OM < 40% to avoid over saturating the CHN Elemental Analyzer during runs. To measure %TN, sediment from each station was transferred into tin capsules, weighed, and the capsules were then carefully folded to ensure no loss of sediment. For %TOC determination, sediment was placed into silver capsules, weighed, and subsequently acidified with the addition of 40 µL of 1.2M hydrochloric acid (HCl). Subsamples were then left to dry for ~1-2 h. Acidification was performed four times (until there was no more apparent bubbling) to ensure the removal of inorganic carbon (Kennedy et al., 2005). The silver capsules were then folded in the same fashion as the tin capsules after a last overnight drying at 60˚C.

CHN determination was carried out using a CE-440 Elemental Analyzer (Exeter Analytical, INC) at the NWAFC. Blanks (empty tin capsules) and standards (Acetanilide, closed tin capsules, 2-3 mg) were incorporated into each CHN run to calibrate and ensure the CHN analyzer was functioning properly.

2.2.3.4 Elemental analysis

Elemental composition was determined using homogenized freeze-dried sediment from each station. All analytical work was performed at the CREAIT ICP-MS trace element lab facility (Memorial University, Canada). For each run, subsamples of ~0.05 to 0.1 g of

sediment were weighed into Savillex® PFA screw cap vials. To ensure decomposition of the organic matter but avoid digestion of the inorganic components, closed-vessel digestion was carried out with 1 mL of distilled 16M HNO_3 + 1 mL of 30% H_2O_2, on a hotplate at ~70°C for ~12 h, in successive steps. Once the organic matter was digested, the sample solutions were dried. Once dry, samples were brought back into solution with 5 mL of distilled 6M HCl to ensure the extraction of trace metals from any remaining organic matter that was not fully digested. Samples were put in the ultrasonic bath for ~1 h and put back on the hotplate at ~100ºC overnight. The following day, samples were removed from the hotplate and weighed once they were cool. Afterwards, sample solutions were transferred into centrifuge tubes and centrifuged for ~30 min. The supernatants were then pipetted out into the respective screw cap vials and put to dry on the hotplate. Samples were then converted to nitrates by adding 0.5 mL of distilled 16M HNO_3 and capped on the hotplate for a few hours, after which they were dried again. In preparation for TE analysis, samples were brought back into solution with 12 mL of distilled 0.2M HNO_3 and further diluted to a total of ~3000-fold.

Elemental analyses were carried out on a Perkin Elmer Elan DCR II Quadrupole Inductively Coupled Plasma Mass Spectrometer, using multi-element external calibration solutions and Sc, Re, Rh and Th as internal standards to monitor for instrumental drift, similarly to the method of Friel et al. (1990). During this study, quality control was assured by processing and analyzing a procedural blank, a mussel reference material (NIST 2977), three duplicate samples and one replicate for each run. Where only one sample was used per station in this analysis, standard deviations were taken from the average of two replicates, per element, from NIST 2977. Trace element concentrations that fell below the

detection limit (<DL), were manually converted to the corresponding methodological detection limit value.

While trace element concentrations in sediments are often normalized against Lithium (Yeats et al., 2005), this approach was not used here given that we did not have enough samples from far-field sites to adequately characterize correlations between Li and other trace elements such as Zn.

2.2.4 Statistical analyses

2.2.4.1 Relationships between sediment characteristics

To explore relationships between sediment characteristics, we first constructed a Pearson correlation matrix in Minitab 17 using parameters measured from the %OM, CHN and elemental analyses. %OM, %TN, %TOC and TOC/TN ratios were selected, as well as the elements P, Ca, Fe, Co, Ni, Zn, Se, Cd, Pb, Cr and Cu. These elements were pre-selected from a larger dataset as they are known markers of aquaculture activity (Hamoutene et al., 2018). Cr and Cu were log(x+1) transformed prior to correlation to remove skewness from the data. Values for Cr and Cu are presented herein as log transformed.

2.2.4.2 Relationships with bacterial mat presence

Relationships between abiotic characters and the presence/absence of white bacterial mats were explored. An unpaired two-tailed *t*-test was performed for each variable to determine if there was a statistically significant difference in the concentration of that particular sediment characteristic between stations with and without bacterial mats. Data were pooled to include both sampling years and all production, fallow and reference

stations. Prior to *t*-test analyses, Levene's and Bonett's tests were run to examine whether variances were equal between pairs and justify the use of parametric tests. A 95% confidence interval was selected for the *t*-test.

In addition, scatterplots representing sediment abiotic concentrations according to distance from cage, with bacterial mat presence or absence highlighted using different station label colours, were constructed using GraphPad Prism 7. For each sediment characteristic, data from all sites and sampling dates were pooled to search for overall trends.

2.2.4.3 Spatial change: relationships with distance to cage

To investigate the relationship between sediment characteristics and distance to cage, scatterplots were first created using GraphPad Prism 7 to visualize concentrations of each parameter at different distances from the cage arrays, with data points representing stations from all transects at both 2016 and 2017 sampling periods. Separate graphs were produced for the production (Fig. 2.2) and fallow site (Fig. 2.3), and stations were labeled by transect name and sampling date. Linear, quadratic (2nd order polynomial) and cubic (3rd order polynomial) regression analyses were performed for each scatterplot, with all data points (transects and sampling dates) pooled, using global nonlinear regression in GraphPad Prism 7. Due to limitations with this program and Minitab 17, *P*-values were reported only for linear regressions (Minitab, 2018). Goodness-of-fit for linear and non-linear regressions were examined using R^2 values. Confidence interval ranges from each regression were also considered when evaluating curve fit.

We then investigated relationships with distance to cage along a single, 160 m-long transect from the fallow site (3S: the longest transect, with the greatest number of successful grab samples in our dataset, Table 2.1) to remove any confounding effects of considering multiple transects simultaneously. Scatterplots were constructed using GraphPad Prism 7, including only data from transect 3S, but combining data from 2016 and 2017. Exceptionally, Zinc data for 2016 and 2017 were considered separately as there was significant difference between sampling years using the Wilcoxon signed-rank test for this parameter only (see below). Then, as described for the initial analysis that examined data from all transects, linear, quadratic and cubic regression models were applied to the 3S data, *P*-values were reported for linear regressions, goodness-of-fit was examined using R^2 values, and confidence intervals were considered.

2.2.4.4 Temporal change at the fallow site (3S transect)

Given that we were not able to repeatedly sample from the same stations along most transects and that most transects were short, we chose to focus on transect 3S from the fallow site (where repeated sampling was achieved at all 6 stations of the transect; Table 2.1) in our examination of differences between sampling years. A Wilcoxon signed-rank test was performed in Minitab 17 to compare sediment characteristics after 24 and 34 months of fallowing at the six stations from transect 3S. At stations where a second grab was obtained in the same year (i.e. the 0 m station from 2016 and the 40 m station from 2017), averages were calculated to balance the test design. These averages were not used when constructing Fig. 2.4 and Table 2.8.

2.3 Results

2.3.1 Relationships between sediment characteristics

Many of the sedimentary characteristics were significantly correlated with one or more of the other characteristics. Notably, %OM showed significant negative correlations with Fe and Co (p= 0.001), Pb (p = 0.01) and Ni (p= 0.05). Further, %OM showed significant positive correlations with P, Ca and Zn (p= 0.001), Cu(log) (p= 0.01) and Cd (p= 0.05).

We reduced the number of variables to be further considered using statistical analysis based on a Pearson auto-correlation level of 0.8 (arbitrarily chosen) (Table 2.3); therefore, %TN, Co, Fe, P, Se and Ni were excluded from further analyses, while %TOC, %OM, Zn, Cd, Pb, Cr (log) and Cu (log) were retained. We also chose to retain TOC/TN because of its direct association with sediment quality. Although Cu(log) and Cd are highly correlated (r= 0.809***), these variables were also considered separately because they are both direct markers of wastes of aquaculture; Cu and Cd are in fish feed (Dean et al., 2007) and Cu is also used in anti-fouling agents (Thomas and Brooks, 2010).

Table 2.2. Pearson correlation matrix coefficients (*r*) for abiotic variables determined from all sediment samples (times and stations) in this study. TOC, total organic carbon; TN, total nitrogen, OM, organic matter. Significant values are denoted as: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

	%TOC	%TN	TOC/TN	%OM	P	Ca	Fe	Co	Ni	Zn	Se	Cd	Pb	Cr (log)
%TN	0.899***													
TOC/TN	0.797***	0.487**												
%OM	0.756***	0.806***	0.487**											
P	0.604***	0.821***	0.150	0.658***										
Ca	0.498**	0.651***	0.142	0.549***	0.877***									
Fe	-0.859***	-0.858***	-0.542***	-0.638***	-0.751***	-0.736***								
Co	-0.888***	-0.894***	-0.576***	-0.686***	-0.756***	-0.713***	0.986***							
Ni	-0.578***	-0.511**	-0.447**	-0.347*	-0.281	-0.338*	0.737***	0.742***						
Zn	0.719***	0.892***	0.298	0.795***	0.916***	0.788***	-0.770***	-0.802***	-0.414**					
Se	-0.538***	-0.478**	-0.367*	-0.282	-0.463**	-0.581***	0.809***	0.740***	0.709***	-0.372*				
Cd	0.056	0.350*	-0.281	0.391*	0.532***	0.364*	-0.078	-0.121	0.213	0.596***	0.323*			
Pb	-0.774***	-0.730***	-0.549***	-0.513**	-0.601***	-0.611***	0.927***	0.920***	0.773***	-0.615***	0.858***	0.112		
Cr (log)	-0.535***	-0.412**	-0.506**	-0.221	-0.182	-0.288	0.672***	0.671***	0.940***	-0.284	0.686***	0.355*	0.745***	
Cu (log)	0.217	0.457**	-0.141	0.498**	0.607***	0.366*	-0.230	-0.271	-0.015	0.679***	0.130	0.809***	-0.042	0.180

Table 2.3. List of variables that are highly correlated with each other.

Selected variable	Highly correlated variable(s)
%TOC	%TN (0.899***) Co (-0.888***) Fe (-0.859***)
%OM	%TN (0.806***)
Zn	P (0.916***) %TN (0.892***) Co (-0.802)
Cd	log Cu (0.809***)
Pb	Fe (0.927***) Co (0.920***) Se (0.858***)
Cr (log)	Ni (0.940***)
Cu (log)	Cd (0.809***)

(>0.80) based on Pearson coefficients (*r)* from Table 2.2.

2.3.2 Relationships with bacterial mats

An unpaired two-tailed *t*-test was conducted to investigate the relationship between the presence/absence of bacterial mats and each selected variable (Table 2.4). The Levene's and Bonett's tests indicated that all variances between pairs were equal supporting our use of this parametric test. Results of the *t*-test indicated that %OM, Zn, Cd and Cu (log) values were significantly higher at sites with bacterial mats.

Mats were mostly found near cage edge and were less frequently observed at 20 m, 40 m, 80 m and 120 m stations. Out of 41 stations, 10 showed no evidence of mats; these stations were located 20 –160 m from cages and at the reference site (> 500 m from cages). At the remaining 31 stations in this study, mats were present regardless of site status and year. With regards to the concentrations of selected sediment variables, mats were found under a broad range of concentrations for all variables and were more prominent near cage edge (Fig. 2.1).

Table 2.4. Statistics for unpaired two-tailed *t*-tests comparing average values for each selected variable in the presence or absence of mats, regardless of sampling year and site status. *n*= number of stations.

Samples Statistics					T-test Statistics		
		Mean	SD	*N*	*t*	*df*	*p*-value (2-tailed)
%TOC	no mats	7.1	11.1	10	-1.91	39	0.063
	mats	15.2	11.7	31			
TOC/TN Ratio	no mats	8.26	2.40	10	0.42	39	0.678
	mats	7.90	2.35	31			
%OM	no mats	13.5	15.9	10	-2.18	39	**0.036**
	mats	26.7	16.9	31			
Zn	no mats	144	160	10	-3.03	39	**0.004**
	mats	370	216	31			
Cd	no mats	0.546	0.307	10	-2.38	39	**0.022**
	mats	0.894	0.426	31			
Pb	no mats	19.60	8.75	10	0.73	39	0.471
	mats	17.26	8.89	31			
Cr(log)	no mats	1.322	0.227	10	-0.67	39	0.507
	mats	1.377	0.224	31			
Cu(log)	no mats	1.452	0.271	10	-2.85	39	**0.007**
	mats	1.690	0.215	31			

Note: *p*-values in bold are considered significant (<0.05). Sediment weights for %TOC and TOC/TN Ratio measured in mg, %OM measured in g and elements measured in ppm.

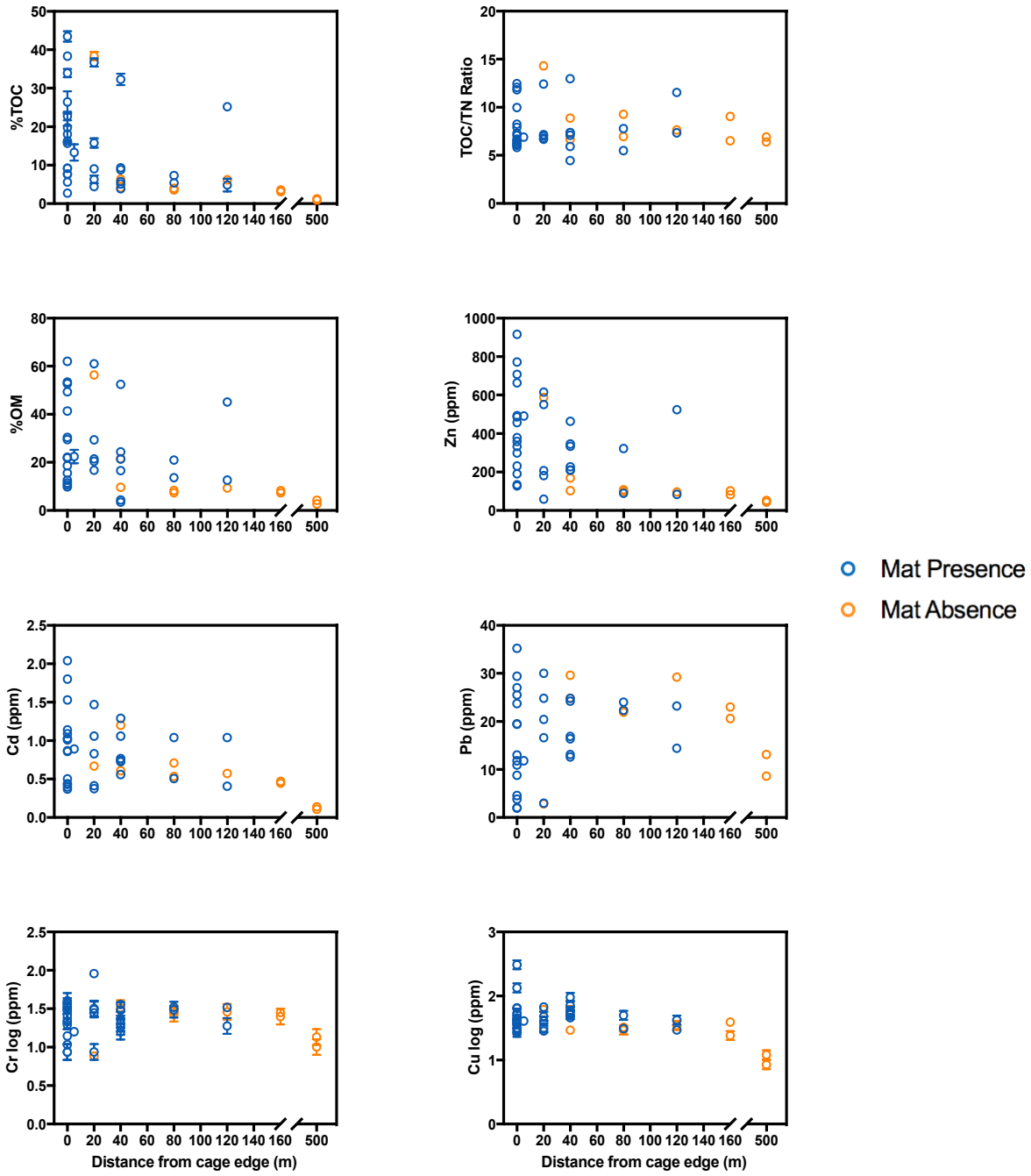
%TOC
TOC/TN Ratio
%OM
Zn (ppm)
Cd (ppm)
Pb (ppm)
Cr log (ppm)
Cu log (ppm)
Distance from cage edge (m)
Distance from cage edge (m)
Mat Presence
Mat Absence

Figure 2.1. Relationship between the concentration of each selected variable (shown by distance to cage) and the presence or absence of bacterial mats. Data are averages ± standard deviations at production, fallow and reference stations (combined), from both 2016 and 2017 sampling years. For some stations, the error bars are shorter than the height of the symbol and are not visible. See Appendix C for information on the number of replicates per station for each variable.

2.3.3 Relationships with distance at the production and fallow sites

For the selected variables %TOC, TOC/TN, %OM, Zn, Cd, Pb, Cr (log) and Cu (log), relationships with distance at both the production and the fallow site (considered separately) were first examined by considering data from all transects. Results of the linear, quadratic and cubic regressions showed that $R2$ values were generally low for both production and fallow sites. The lowest $R2$ values came from linear regressions, ranging from 4.54e-007 for Cd to 0.1855 for Zn during production (Fig. 2.2, Table 2.5) and 0.003734 for Pb to 0.3143 for Cd during fallow (Fig. 2.3, Table 2.6). Goodness-of-fit tended to increase from linear to quadratic, with cubic regressions providing the best fit in each plot. $R2$-values from production site scatterplots using a cubic regression ranged from 0.0003326 for Cd to 0.4143 for %TOC, while values from fallow site scatterplots ranged from 0.01904 for Pb to 0.3467 for Cd. Linear regressions were not statistically significant for any of the variables at the production site (Table 2.5), but at the fallow site, *p*-values indicated that Cd and Cu(log) showed statistically significant linear regressions, at 0.0044 and 0.0136, respectively (Table 2.6). When examining confidence intervals from each regression, all fell within a narrow, acceptable CI range according to the program.

When examining the range of values for each variable across distance, the range at the production site was large, especially at stations sampled at cage edge (e.g. %TOC= 2.72 – 43.5; Fig. 2.2). For the fallowing site, values tended to be similar along transects (Fig. 2.3). However, there were a few obvious outliers for some of the parameters, most notably, stations along the 3S transect sampled at 40 m and 120 m.

2.3.4 Spatial and temporal patterns at the fallow site (3S transect)

The relationship between parameters and distance to cage was also investigated along the longest fallow transect (3S). Prior to examining the effect of distance, a Wilcoxon signed-rank test was run to examine whether median values of variables differed significantly between years, which would warrant a separate analysis of 2016 and 2017 data. The Wilcoxon signed-rank test indicated that there was a statistically significant difference in Zn along the 3S transect between 2016 and 2017 sampling years, W=0.0, p= 0.036 (Table 2.7). As no significant difference was found for the other seven variables, data from both years were pooled for all but Zn, and represented on scatterplots (Fig. 2.4). Linear, quadratic and cubic regression models were applied to each scatterplot (Table 2.8). As was observed for the analysis that included all transects, R^2 values were lowest for linear models and highest for cubic regressions. Considering the combined 2016 and 2017 data, R^2 values were generally low; the highest values were for the cubic regression of Cd data (R^2= 0.3613). For Zn data separated by year, R^2 values (cubic regression) were much higher in 2017 (R^2= 0.6065) than in 2016 (R^2= 0.2742). P-values indicated that none of the regressions were statistically significant under a linear model (Table 2.8).

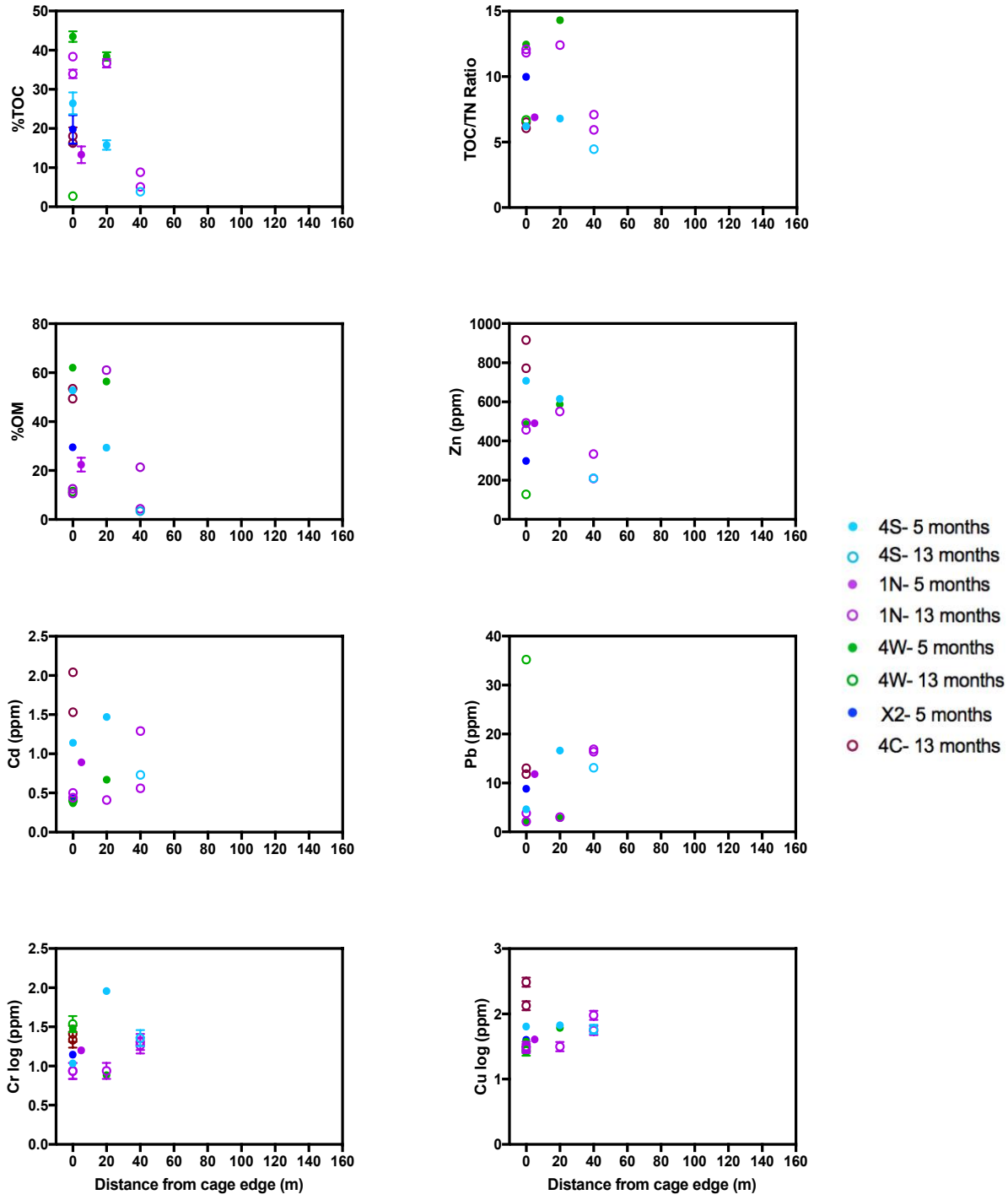
%TOC
TOC/TN Ratio
%OM
Zn (ppm)
Cd (ppm)
Pb (ppm)
Cr log (ppm)
Cu log (ppm)
Distance from cage edge (m)
Distance from cage edge (m)
4S- 5 months
4S- 13 months
1N- 5 months
1N- 13 months
4W- 5 months
4W- 13 months
X2- 5 months
4C- 13 months

Figure 2.2. Relationship between selected variables and distance to cage at the production site, after 5 months (2016) and 13 months (2017) of production. Data points represent averages from a given station, with error bars showing standard deviations. For some points, the error bars are shorter than the height of the symbol and are not visible. See Appendix C for information on the number of replicates per station for each variable.

Table 2.5. Regression model statistics to explore relationships between selected variables and distance to cage during production. Data analyzed are represented graphically in Fig. 2.2. All data points (transects and sampling dates) were pooled.

	%TOC	TOC/TN Ratio	%OM	Zn	Cd	Pb	Cr (log)	Cu (log)
Linear Regression	y= 25.89 - 0.3642x R^2= 0.1842 *p*= 0.1104	y= 9.291 - 0.05228x R^2= 0.07352 *p*= 0.3283	y= 37.63 - 0.4559x R^2= 0.1182 *p*= 0.2097	y= 555.8 - 5.837x R^2= 0.1855 *p*= 0.1090	y=0.8583 - 2.137e-005x R^2= 4.54e-007 *p*= 0.9981	y= 9.587 - 0.09784x R^2= 0.0332 *p*= 0.5157	y= 1.223 + 0.002062x R^2= 0.01359 *p*= 0.6791	y= 1.726 - 0.001743x R^2= 0.01012 *p*= 0.7213
Quadratic Regression	y= 23.73 + 0.8857x - 0.03299x^2 R^2= 0.3327	y= 8.733 + 0.2703x - 0.008514x^2 R^2= 0.2652	y= 33.67 + 1.833x - 0.06042x^2 R^2= 0.3221	y= 525.7 + 11.52x - 0.4581x^2 R^2= 0.2979	y= 0.8589 - 0.0004159x 1.041e-005x^2 R^2= 1.105e-005	y= 10.41- 0.3749x + 0.01248x^2 R^2= 0.08629	y= 1.225 + 0.00094x + 2.961e-005x^2 R^2= 0.01386	y= 1.742 - 0.007637x + 0.0002476x^2 R^2= 0.03019
Cubic Regression	y= 24.88 - 3.769x + 0.3216x^2 - 0.00598x^3 R^2= 0.4143	y= 8.977 - 0.7203x + 0.06694x^2 - 0.001273x^3 R^2= 0.3368	y= 35.22 - 4.446x + 0.4178x^2 - 0.008066x^3 R^2= 0.3828	y= 532.4 - 15.36x + 1.589x^2 - 0.03453x^3 R^2= 0.3085	y= 0.8562 + 0.01051x - 0.0008217x^2 + 1.403e-005x^3 R^2= 0.0003326	y= 10.16 + 0.6079x - 0.06238x^2 + 0.001263x^3 R^2= 0.09537	y= 1.228 - -0.008902x + 0.0007793x^2 - 1.264e-005x^3 R^2= 0.0147	y= 1.751 - 0.04169x + 0.002841x^2 - 4.374e-005x^3 R^2= 0.04066

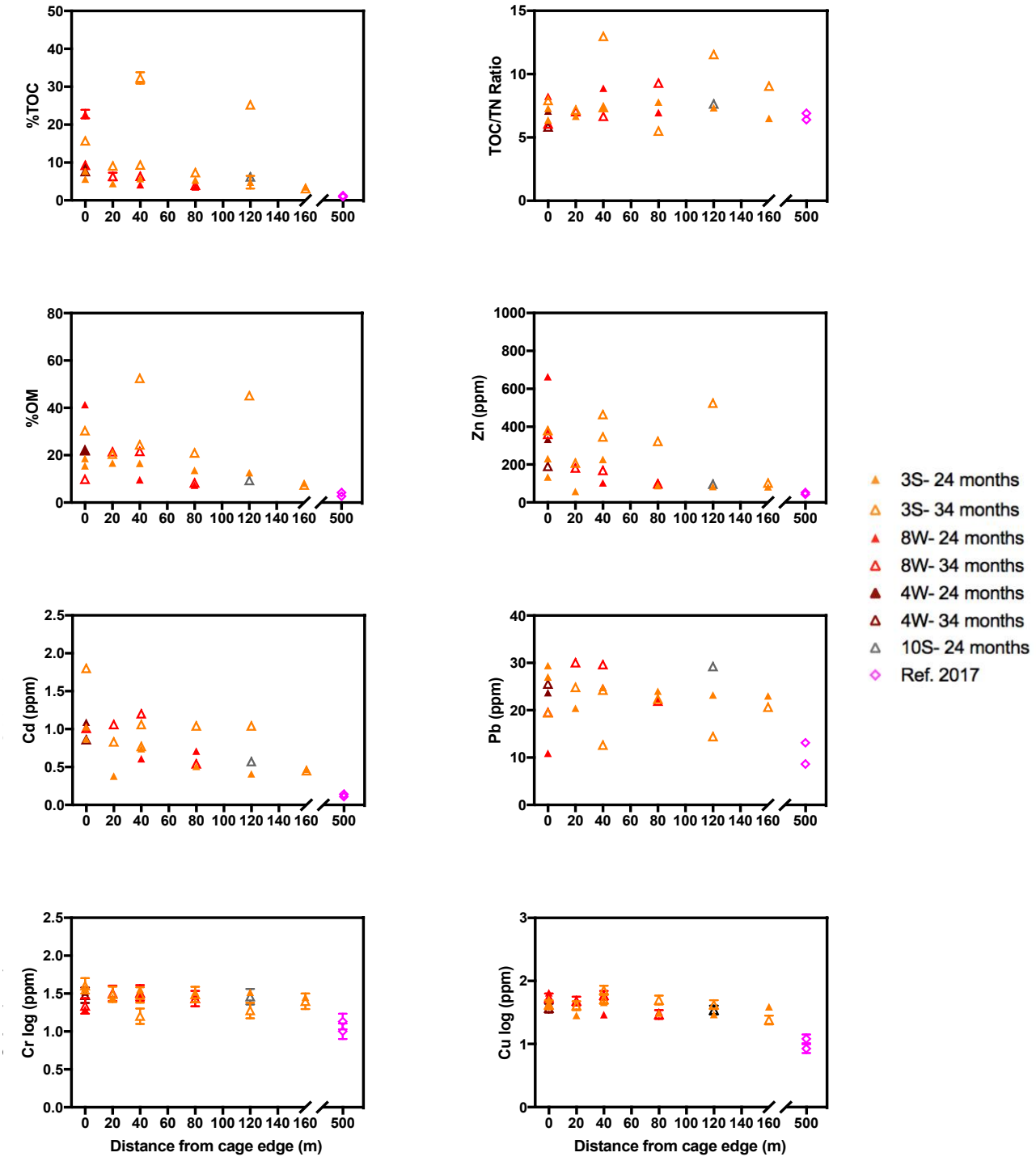
%TOC
TOC/TN Ratio
%OM
Zn (ppm)
Cd (ppm)
Pb (ppm)
Cr log (ppm)
Cu log (ppm)
Distance from cage edge (m)
Distance from cage edge (m)
3S- 24 months
3S- 34 months
8W- 24 months
8W- 34 months
4W- 24 months
4W- 34 months
10S- 24 months
Ref. 2017

Figure 2.3. Relationship between selected variables and distance to cage at the fallow site, after 24 months (2016) and 34 months (2017) of fallowing. Data points represent averages for a given station, with error bars showing standard deviations. For some stations, the error bars are shorter than the height of the symbol and are not visible. See Appendix C for information on the number of replicates per station for each variable.

Table 2.6. Regression model statistics to explore relationships between each selected variable and distance to cage during fallowing. Data analyzed are represented graphically in Fig. 2.3. *p*-values in bold are considered significant (<0.05). All data points (transects and sampling dates) were pooled apart from the reference site (> 500 m), which was excluded from these analyses.

	%TOC	TOC/TN Ratio	%OM	Zn	Cd	Pb	Cr (log)	Cu (log)
Linear Regression	y= 10.55 - 0.02751x R^2= 0.0371 *p*= 0.3672	y= 7.271 - 0.007868x R^2= 0.05803 *p*= 0.2568	y= 23.38 - 0.0676x R^2= 0.08629 *p*= 0.1636	y= 286.8 - 1.06x R^2= 0.1192 *p*= 0.0984	y=1.019 - 0.003481x R^2= 0.3143 *p*= **0.0044**	y= 23.12 - 0.005833x R^2= 0.003734 *p*= 0.7767	y= 1.48 - 0.0003668x R^2= 0.03867 *p*= 0.3570	y= 1.673 - 0.001165x R^2= 0.2465 *p*= **0.0136**
Quadratic Regression	y= 10.43 - 0.01941x - 5.682e-005x^2 R^2= 0.03744	y= 7.004 + 0.02604x - 0.0001275x^2 R^2= 0.09041	y= 22.68 - 0.01994x - 0.0003344x^2 R^2= 0.09077	y= 298 - 1.821x + 0.005341x^2 R^2= 0.1257	y= 1.046 - 0.005299x + 1.276e-005x^2 R^2= 0.3232	y= 22.77 + 0.01758x - 0.0001643x^2 R^2= 0.01003	y= 1.479 - 0.0003194x - 3.325e-007x^2 R^2= 0.03874	y= 1.671 - 0.001023x - 9.93e-007x^2 R^2= 0.2469
Cubic Regression	y= 11.09 - 0.1399x + 0.002222x^2 - 1.004e-005x^3 R^2= 0.0572	y= 6.955 + 0.03503x - 0.0002974x^2 + 7.488e-007x^3 R^2= 0.09251	y= 23.16 - 0.1079x + 0.001329x^2 - 7.328e-006x^3 R^2= 0.09483	y= 317.9 - 5.455x + 0.07405x^2 - 0.0003028x^3 R^2= 0.1646	y= 1.077 - 0.01101x + 0.0001207x^2 - 4.756e-007x^3 R^2= 0.3467	y= 22.48 + 0.07198x - 0.001193x^2 + 4.532e-006x^3 R^2= 0.01904	y= 1.48 - 0.0005399x + 3.835e-006x^2 - 1.837e-008x^3 R^2= 0.03913	y= 1.666 - 9.414e-005x - 1.855e-005x^2 + 7.739e-007x^3 R^2= 0.2512

Table 2.7. Results of the Wilcoxon signed-rank test comparing measurements of selected variables taken along the longest fallow transect (3S) in 2016 and in 2017.

	(n)	Wilcoxon Statistic (*W*)	*p*-value
%TOC	6	1.0	0.059
TOC/TN Ratio	6	3.0	0.142
%OM	6	1.0	0.059
Zn	6	0.0	**0.036**
Cd	6	1.0	0.059
Pb	6	18.0	0.142
Cr (log)	6	16.0	0.295
Cu (log)	6	7.0	0.529

Note: Bolded terms indicate a significant difference between sampling years at $p<0.05$. *n* represents *n* after the median difference between variables was calculated. 14 stations were used in total in this test with averages calculated from two stations at 0 m in 2016 and two stations at 40 m in 2017 to balance the test design.

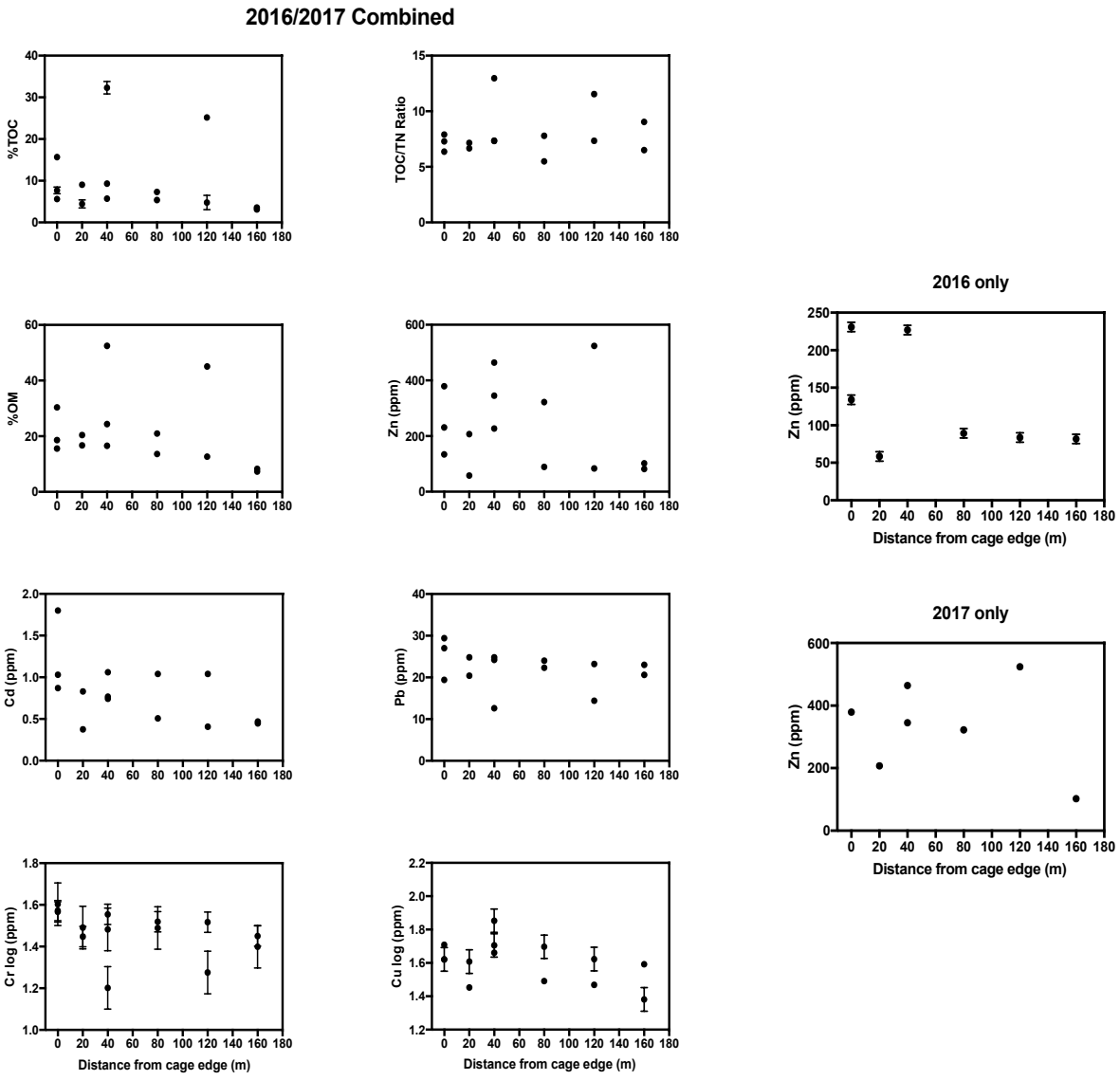

Figure 2.4. Relationship between selected variables and distance to cage along the longest fallow transect (3S), with 2016 and 2017 stations combined (left panel) and separated by year for Zn (right panel). Data points represent averages for a given station, with error bars showing standard deviations. For some stations, the error bars are shorter than the height of the symbol and are not visible. See Appendix C for information on the number of replicates per station for each variable.

Table 2.8. Regression model statistics to explore relationships between each selected variable and distance to cage along the longest fallow transect (3S) for both sampling years, with 2016 and 2017 data combined, except for Zn (separated based on Wilcoxon signed-rank test results shown in Table 2.7). *n*= number of stations. Data analyzed are represented graphically in Fig. 2.4.

	%TOC	TOC/TN Ratio	%OM	Zn	Zn	Zn	Cd	Pb	Cr (log)	Cu (log)
Sampling Year	2016/2017	2016/2017	2016/2017	2016/2017	2016	2017	2016/2017	2016/2017	2016/2017	2016/2017
n	14	14	14	14	7	7	14	14	14	14
Linear Regression	y= 11.26 - 0.02095x R^2= 0.01927 *p*= 0.6360	y= 7.567 + 0.005503x R^2= 0.02428 *p*= 0.5948	y= 25.12 - 0.05517x R^2= 0.05937 *p*= 0.4012	y= 264.4 - 0.5151x R^2= 0.03763 *p*= 0.5064	y= 165 - 0.5946x R^2= 0.2656 *p*= 0.2365	y= 365.3 - 0.6327x R^2= 0.06319 *p*= 0.5866	y=1.025 - 0.003362x R^2= 0.2566 *p*= 0.0645	y= 23.51 - 0.02163x R^2= 0.0764 *p*= 0.3388	y= 1.517 - 0.0007642x R^2= 0.1519 *p*= 0.1683	y= 1.667 - 0.0009638x R^2= 0.2 *p*= 0.1088
Quadratic Regression	y= 8.868 + 0.1149x - 0.0008746x^2 R^2= 0.08435	y= 7.219 + 0.02528x - 0.0001273x^2 R^2= 0.04944	y= 20.39 + 0.213x - 0.001726x^2 R^2= 0.1719	y= 214.2 + 2.333x - 0.01833x^2 R^2= 0.1299	y= 169.1 - 0.8831x + 0.001911x^2 R^2= 0.2706	y= 275.1 + 4.098x - 0.02935x^2 R^2= 0.3269	y= 1.066 - 0.005693x + 1.501e-005x^2 R^2= 0.2666	y= 24.68 - 0.08831x + 0.0004291x^2 R^2= 0.1346	y= 1.547 - 0.002462x + 1.092e-005x^2 R^2= 0.2121	y= 1.635 + 0.0008396x - 1.161e-005x^2 R^2= 0.2562
Cubic Regression	y= 9.515 + 0.02509x + 0.0007395x^2 - 6.895e-006x^3 R^2= 0.09215	y= 7.196 + 0.02843x - 0.0001839x^2 – 2.421-007x^3\ R^2= 0.04962	y= 21.51 + 0.05713x + 0.001074x^2 - 1.196e-005x^3\ R^2= 0.1823	y= 231.8 - 0.1088x + 0.02553x^2 - 0.0001874x^3 R^2= 0.1486	y= 166.4 - 0.3851x - 0.007013x^2 + 3.837e-005x^3 R^2= 0.2742	y= 361.9 - 4.893x + 0.1268x^2 - 0.000661x^3 R^2= 0.6065	y= 1.165 - 0.01946x + 0.0002623x^2 - 1.056e-006x^3 R^2= 0.3613	y= 24.89 - 0.1169x + 0.0009429x^2 - 2.195e-006x^3 R^2= 0.1376	y= 1.569 - 0.005457x + 6.472e^e-005x^2 - 2.298e-007x^3 R^2= 0.2635	y= 1.622 + 0.002571x - 4.271e^e-005x^2 + 1.329e-007x^3 R^2= 0.2704

2.4 Discussion

Previous studies have reported changes in sediment characteristics at NL salmon farm sites based on %OM, sulfide and redox measurements (Anderson et al., 2005, Hamoutene, 2014). Although sediment characters may constitute valuable metrics for monitoring the benthic effects of aquaculture, the assessment of organic enrichment at hard-bottom sites in NL relies on standard video monitoring protocols due to the low success rate of grab sampling (Hamoutene, 2014). Here, we aimed to relate a broader suite of sediment (or flocculent matter) characteristics with aquaculture activity in this region, during both production and fallow states, and attempt to narrow down the sedimentary conditions under which bacterial mats are observed. It is also the first study in the region documenting the presence of potential pollutants linked to aquaculture such as Zn and Cu, and their relationships with time and space.

Although grab sampling was challenging, we successfully obtained 15 grab samples from a production site and 24 grabs samples from a fallow site during this two-year study. Results indicated that hard-bottom aquaculture sites off the south coast of NL had been affected by organic loading due to salmon farming. This was evident by the high amount of OM near and around cages, regardless of site status, compared to lower amounts at more distant locations. Over the two years of this study, the fallow site appeared to remain in the early stages of recovery, even after 4 years of fallowing. Relationships between selected sedimentary variables and the presence or absence of white bacterial mats indicated that the latter were associated with organic-rich areas.

2.4.1 Relationships between sediment variables

Through a correlation matrix analysis, we explored the relationships between measured variables within substrates collected at hard-bottom NL production, fallow and reference sites. Many significant correlations between sediment components were found. The measures of organic content (%TOC, %TN and %OM) were similarly correlated with some of the elements quantified, which is not surprising as these three variables were all highly correlated with one another (R>0.75). Previous studies showed that TOC and OM were significantly, positively correlated with each other in benthic sediments (Agah et al., 2013), due to organic carbon typically being abundant in organically enriched sediment, especially here where OM enrichment is coming from fish pellets. In our study, OM showed significant positive correlations with P, Ca, Zn, Cu and Cd. High concentrations of these elements are consistently found at aquaculture sites and reflect organic enrichment; Sutherland et al. (2007) reported high abundances of Zn and Cd in farmed fish feces, while collectively, P, Ca, Zn, Cu and Cd have been found in fish feed, with Zn, P and Cu being particularly abundant along with organic carbon and nitrogen (Chou et al., 2004; Olsen et al., 2008). Zn and Cu correlations may also be a result of biofouling paint accumulated into the sediment as seen in other studies (Macleod et al., 2014; Nikolaou et al., 2014). Significant negative correlations exist between OM and Fe, Co, Pb and Ni, suggesting that these elements are found in low concentrations in fish feed. Similar trends were found between high OM and low Fe concentrations at impacted stations, where Fe tended to decrease as sediments became less oxygenated (Chou et al., 2002, 2004). It is unclear why Cr showed no significant relationship with OM but had a strong significant correlation with %TOC. It is notable that Cr has been found in higher concentrations in fish manure than in other types of livestock manures (Naylor et al., 1999). Overall, these results indicate that

fish feces and uneaten fish pellets heavily influence the composition of sediments, including flocculent matter, near cages.

2.4.2 Associations between sediment characteristics and bacterial mats

Drop-camera video at production, fallow and reference sites, showed that mats were found at 31 out of 41 stations. Exploration of trends between bacterial mats and sediment characteristics revealed a significant difference in %OM, Zn, Cd and Cu (log) between stations with and without bacterial mats through t-test analyses, indicating that these factors may play an important role for mat-forming bacteria and that bacterial mat presence can be directly linked to aquaculture waste. Spatial and temporal trends were also investigated. Mats were found at every station sampled at cage edge (0 m), becoming less frequent with distance. These results indicate a strong relationship between mats and organically enriched areas, which has been reported in other studies (Crawford et al., 2001; Clement et al., 2010; Hamoutene et al., 2015). This was further illustrated with the absence of mats at stations farther away (160 and 500 m). Surprisingly, ranges of concentrations for the selected variables at stations where mats were found were broad; for example, mats were found between 9.8 and 62.0 %OM. At the reference station (where no mats were observed), OM concentrations ranged between 2.7 and 4.3 %. There might be an OM threshold beneath which bacterial mats cannot be visible at the benthic interface (somewhere between 4.3 and 9.8 %OM, with oxygen may still be in sufficiently high concentration at the benthic surface to inhibit mat development), although mat-forming bacteria could still be present beneath the sediment surface under those conditions. Mats were present at 17 out of 24 fallowed stations (data not shown) even after 3 years of fallow, which is not surprising since OM remained elevated (>7.3 %) at stations from the fallow site. At NL aquaculture sites,

bacterial mats are dominated by *Prevotella*, *Meniscus* and *Odoribacter* (Verhoeven et al., 2016). As members of those three genera are typically associated with elevated levels of organic waste, it is not surprising that bacterial mats form where %OM, Zn, Cd and Cu (log) are elevated, as they are common markers of aquaculture.

2.4.3 Relationships with site status and distance

Scatterplots and regression analyses revealed interesting results when exploring relationships between selected sediment variables and distance from cage edge. In both production and fallow sites, we saw the highest concentrations of most variables at cage edge (0 m). Higher levels of all eight variables were noted at the production site, which at the time of sampling had received a continuous influx of organic waste due to active farming. The high concentrations of flocculent matter, as evident by high %OM concentrations near cage edge and video evidence, agrees with other studies (Karakassis et al., 2000; Sarà et al., 2004; Porrello et al., 2005; Corner et al., 2006). Concentrations did not show any obvious linear, quadratic or cubic spatial relationship at the production site, as evidenced by scatterplot arrays; regression analyses confirmed this as R^2 values were very low. This is not surprising as stations along different transects were most likely experiencing different levels of organic loading due to variations in current directionality and strength in this oceanographically complex system (Hamoutene et al., 2015, 2016). The sites also experienced multiple cycles of production as well as cage displacement, which may have affected deposition within 40 m from cage edges. However, it should be noted that the dataset from the production site was limited due to the difficulty in obtaining successful grab samples along the transects, especially at distances greater than 40 m where there was less sediment/flocculent matter to be sampled, and where depths were > 100 m. With a greater

spatial range, we would expect organic enrichment to decrease with distance from cages, as has been well-documented in other studies where fish waste is typically dispersed along a gradient up to ~50 m from cages through local currents (Lumb, 1989; Ye et al., 1991; Chou et al., 2002; Carroll et al., 2003). Notably, resuspension events are episodic in NL (Hamoutene et al., 2016) and mainly associated with winter storms (Anderson et al., 2005), so flocculent matter distribution may mainly reflect its initial deposition. Nevertheless, we expect that decreases in OM content away from the cage (point source) are unlikely to be linear or to show similar rates of decrease along all transects. At the fallow site, concentrations of the variables measured were generally lower than at the production site, and showed little to no strong relationship with distance from cage edge, as evidenced by the regression analyses. This was also the case when scatterplot arrays and regressions were applied to the longest transect (3S), exclusively. Along this transect, it appeared that aquaculture wastes had dispersed up to 160 m from cages, based on similar concentrations of variables; such dispersal is not unusual as 'far-field' effects have been measured at distances greater than several hundred meters (Hargrave, 2003; Yokoyama et al., 2006). Concentrations of Zn, Ca and Cu in sediments are also influenced by redox conditions, which control the solubility of these metals and their likelihood to become lost to overlying waters (Dean et al., 2007).

Because the production and fallow sites examined here are in different bays along the South coast of NL, we must be cautious to make direct comparisons of organic enrichment concentrations and spatial dynamics as different factors such as farming intensity, age of site and dispersion rates could play a role in resulting patterns. Aquaculture sites in NL are located in small coves and show considerable variability in terms of substrates, currents and species diversity at the local scale (Hamoutene et al., 2017). Nevertheless, we expected that

lower concentrations of aquaculture-linked abiotic markers would be present in sediments from the fallow site, as a direct result of remediation (Keeley et al., 2015). In fact, maximum concentrations at the fallow site were lower for all 8 variables when compared to the production site. However, concentrations of %TOC, %TN, %OM, Zn, Pb, Cr and Cu at the fallow site were also elevated when compared to the reference site, suggesting that this site has not made a full recovery. Elevated levels of Cu and Zn have also been reported at other fish farms across Canada according to Sutherland and Yeats (2011). Sediment samples collected from finfish sites in Broughton Archipelago, British Columbia and Letang Inlet, southwest New Brunswick indicated that Cu and Zn (as well as molybdenum (Mo) and P) were correlated with organic enrichment (i.e. organic matter) during both production and fallow periods, where elevated levels were also contributed to fish feed, fish feces and the use of anti-fouling paint. Sutherland et al. (2007) also reported similar spatial relationships to this study, as Cu and Zn were found in high abundance between 0-30 m from cages in Broughton Archipelago.

2.4.4 Temporal patterns at the fallow site (3S transect)

The Wilcoxon signed-rank test allowed us to explore differences between years along the longest transect (3S). These results suggested that for most of the selected variables, concentrations did not change over a 10-month remediation period, apart from Zn (p= 0.036). Interestingly, Zn concentrations increased with a mean average of 129 to 335 ppm from 2016 to 2017, respectively. This was unexpected as this site was in fallow during the 2016 and 2017 sampling and thus was not receiving Zn through particulate organic matter (POM) via aquaculture activity. This increase could be due to inputs from other

anthropogenic sources (Burton et al., 2005); however a larger sample size is needed to draw conclusions.

Scatterplot arrays also suggested that concentrations were not changing over time (from one year to the next) at the fallow and production site, when considering all stations (Fig. 2.2 & 2.3). Increases in organic enrichment at the production site would be expected as more SOM accumulates at the seafloor, but concentrations may be remaining stable due to continual remineralization and possibly resuspension. Neofitou et al. (2010) also saw no significant change of OM and TOC during production (after 9 months) at a fish farm in the Mediterranean Sea. Results from the fallow site indicated that a 10-month period is not long enough to show statistically significant signs of recovery in this region, even for a site that was out of production for 3 years (at the time of the last sampling). Other studies have reported diverse rates of recovery; from weeks (Ritz et al., 1989), to 6 months (Brooks et al., 2003), to >5 years (Wan Hussin et al., 2012; Keeley et al., 2014). Differences in recovery rates are dependent on various factors including site location and age, farming intensity, and dispersion rates, and may also vary according to the sediment marker considered (Keeley et al., 2014). Results from our fallow site shows that the site is still impacted and suggest that recovery will be slower than at high-flow and/or less impacted fallow sites.

2.4.5 Conclusions

As finfish aquaculture expands due to a higher demand for fish product, impacts on the surrounding benthic environment will also rise. Sediment at soft-bottom aquaculture sites have been described in literature but studies of sedimentary deposits at hard-bottom dominated sites, especially in NL, are lacking.

This study provided a first characterization of organically enriched sediment beneath aquaculture sites in NL at different times (snapshots) during both production and fallow periods and on spatial and temporal scales. The relationships between bacterial mats and flocculent matter composition were also explored in greater detail than was done previously. We uncovered correlations between measured variables that were related to aquaculture production (fish feed composition and possibly biofouling paint), and that could help inform the future development of additional abiotic markers of aquaculture at the seafloor for monitoring purposes. Relationships between bacterial mat presence and abiotic features suggest causal (for OM) and potentially correlative relationships (for Zn, Cd and Cu), and could help refine our understanding of the abiotic conditions under which these visual indicators are present. Our results highlight the complex patterns of flocculent matter accumulation at the seafloor and the slow rate of natural remediation during fallowing in our region. Future studies should look more closely at the composition of bacterial communities within flocculent matter to further understand biological processes at these sites.

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2.6 Appendices

Appendix A: Maps of sampling transects from production and fallow sites

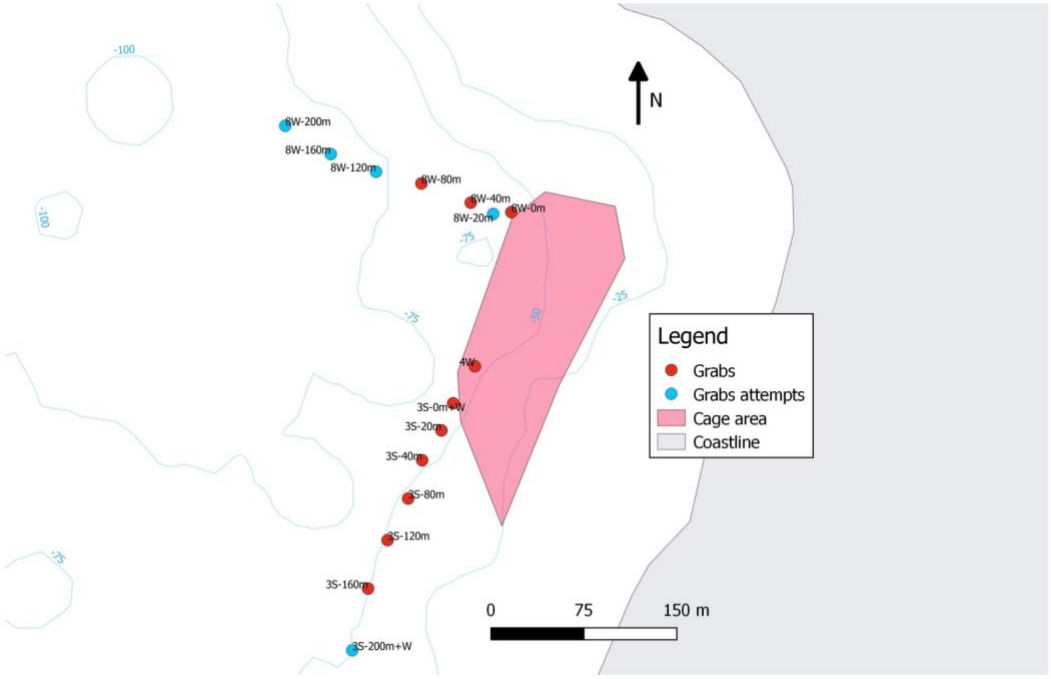

Figure A.1. Fallow site grab sampling along transects in 2016.

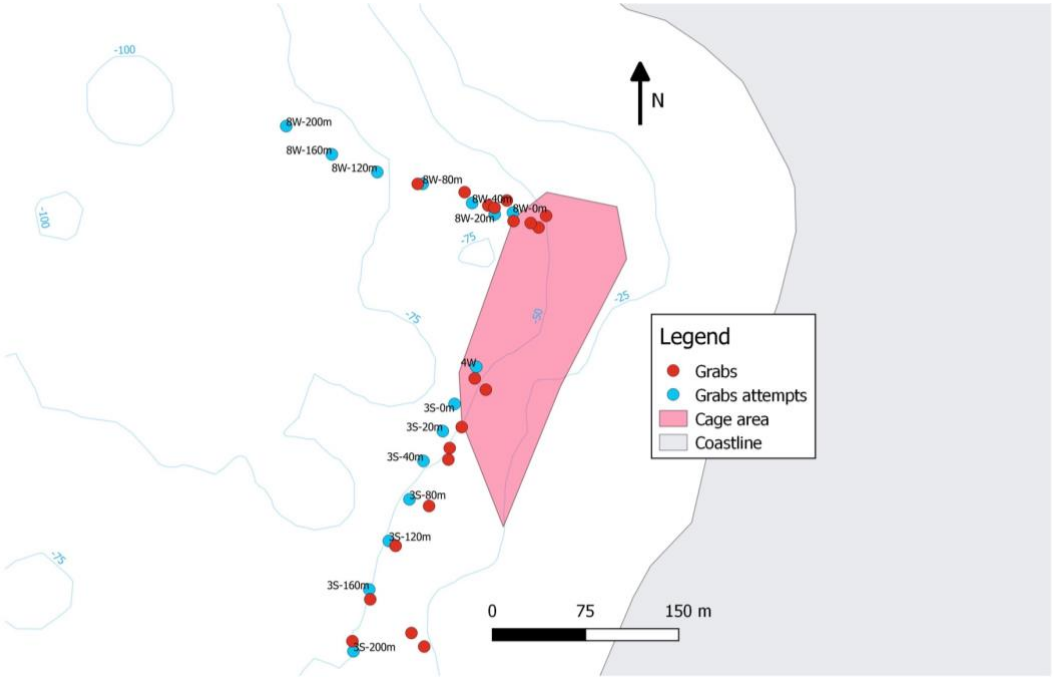

Figure A.2. Fallow site grab sampling along transects in 2017.

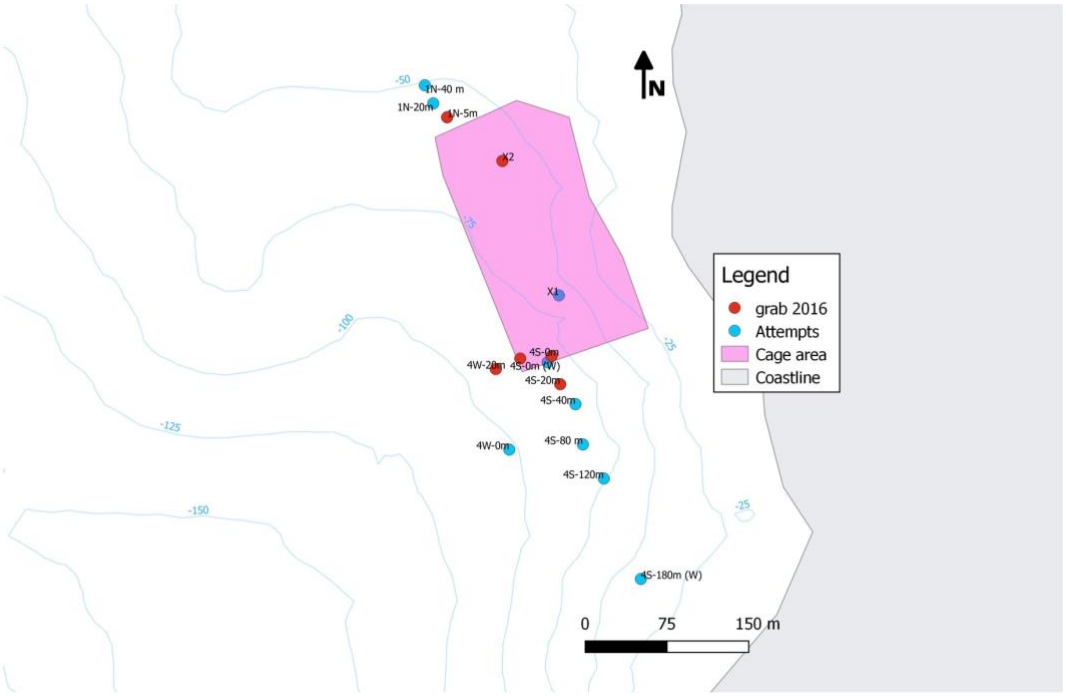

Figure A.3. Production site grab sampling along transects in 2016.

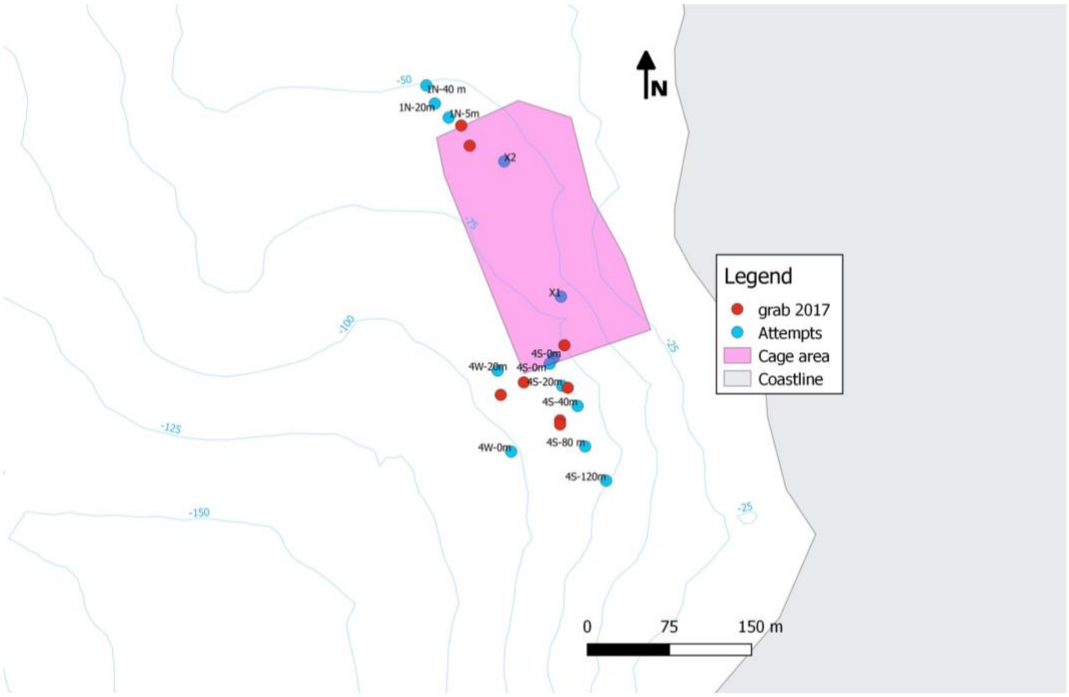

Figure A.4. Production site grab sampling along transects in 2017.

Appendix B: Extracted images from drop-camera video illustrating the presence and absence of flocculent matter on the seafloor

Figure B.1. Example of a station where flocculent matter is present. Station is 4W from the fallow site in 2016.

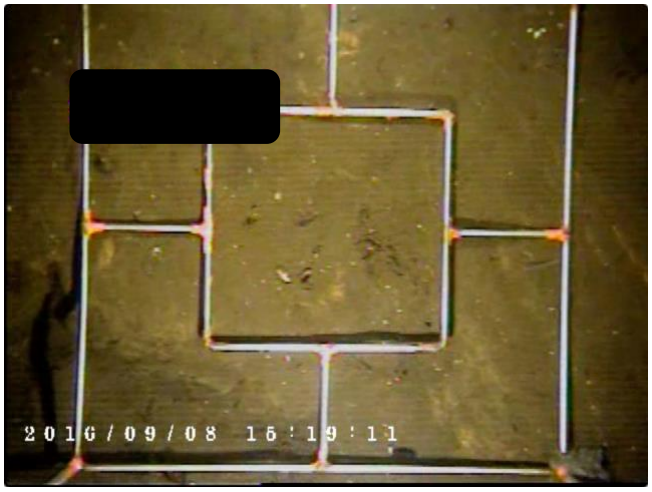

Figure B.2. Example of a station where flocculent matter is absent. Station is 4W-20 from the production site in 2016.

Appendix C: Station data

Table C.1. Description of station grab samples and the number of replicates used per analysis.

Station #	Site Status	Distance from cage (m)	Transect	Sampling Year	%TOC	%TN	TOC/TN Ratio	%OM	Zn	Cd	Pb	Cr	Cu
1	fallow	0	3S	2016	4	2	1	2	1	1	1	1	1
2	fallow	0	3S	2016	2	2	1	2	1	1	1	1	1
3	fallow	20	3S	2016	2	2	1	2	1	1	1	1	1
4	fallow	40	3S	2016	2	2	1	2	1	1	1	1	1
5	fallow	80	3S	2016	4	2	1	2	1	1	1	1	1
6	fallow	120	3S	2016	2	2	1	2	1	1	1	1	1
7	fallow	160	3S	2016	2	10	1	2	1	1	1	1	1
8	fallow	0	8W	2016	5	2	1	2	1	1	1	1	1
9	fallow	40	8W	2016	1	2	1	2	1	1	1	1	1
10	fallow	80	8W	2016	2	2	1	2	1	1	1	1	1
11	fallow	0	4W	2016	3	2	1	2	1	1	1	1	1
14	production	20	4S	2016	5	2	1	2	1	1	1	1	1
15	production	0	X2	2016	3	2	1	2	1	1	1	1	1
16	production	5	1N	2016	3	1	1	2	1	1	1	1	1
21	production	0	4W	2016	3	2	1	2	1	1	1	1	1
22	production	0	4S	2016	2	2	1	2	1	1	1	1	1
23	production	20	4W	2016	2	2	1	2	1	1	1	1	1
29.1	reference	500	Ref	2017	2	2	1	1	1	1	1	1	1
29.2	reference	500	Ref	2017	2	2	1	1	1	1	1	1	1
30	fallow	0	4W	2017	2	2	1	1	1	1	1	1	1
31	fallow	0	3S	2017	2	2	1	1	1	1	1	1	1
32	fallow	20	3S	2017	2	2	1	1	1	1	1	1	1
33	fallow	40	3S	2017	2	2	1	1	1	1	1	1	1

34	fallow	40	3S	2017	2	3	1	1	1	1	1	1	1
35	fallow	80	3S	2017	2	4	1	1	1	1	1	1	1
36	fallow	120	3S	2017	2	3	1	1	1	1	1	1	1
37	fallow	160	3S	2017	2	2	1	1	1	1	1	1	1
38	fallow	80	8W	2017	2	2	1	1	1	1	1	1	1
39	fallow	40	8W	2017	2	2	1	1	1	1	1	1	1
40	fallow	20	8W	2017	2	2	1	1	1	1	1	1	1
41	fallow	0	8W	2017	2	2	1	1	1	1	1	1	1
42	fallow	120	10S	2017	2	2	1	1	1	1	1	1	1
43	production	0	1N	2017	2	2	1	1	1	1	1	1	1
44	production	0	1N	2017	2	2	1	1	1	1	1	1	1
45	production	20	1N	2017	2	3	1	1	1	1	1	1	1
46.1	production	40	1N	2017	2	3	1	1	1	1	1	1	1
46.2	production	40	1N	2017	2	3	1	1	1	1	1	1	1
47	production	40	4S	2017	2	3	1	1	1	1	1	1	1
48	production	0	Center	2017	2	2	1	1	1	1	1	1	1
49	production	0	Center	2017	2	2	1	1	1	1	1	1	1
53	production	0	4W	2017	2	2	1	1	1	1	1	1	1

Chapter 3. Relationships between benthic bacterial communities and sediment characteristics at Newfoundland aquaculture sites during production and fallow

3.1 Introduction

Aquaculture is experiencing considerable growth, with over half of the fish consumed globally now being produced at farms (FAO, 2016). As the global human population increases, the demand for farmed fish is expected to continue to rise (Holmer et al., 2008). Canadian aquaculture makes an important contribution to this market, placing 4th among salmon producers worldwide (DFO, 2013). The aquaculture industry in Newfoundland (NL) accounts for 15% of the total production volume in Canada (DFO, 2013) with most salmon farming established along the south coast of the province, in the Coast of Bays region. This area is characterized by deep sheltered bays and hard-bottom substrates, which are made up of mostly of boulders, rock and cobble with little natural sediment (Anderson et al., 2005; Hamoutene et al., 2013). Salmonid production has seen a tremendous increase in NL recently: notably, a 229.2% increase was recorded between 2014 and 2015, with nearly 20,000 metric tonnes being produced in the latter year (NLDFLR, 2019). Salmon are kept in suspended net pens over water greater than 30 m throughout a 1 to 2-year growth period and are fed fish pellets until harvested, at which point NL producers typically apply a fallow period lasting a minimum of 7 months per site.

3.1.1 Organic enrichment and current monitoring practices

With the growth of finfish aquaculture, certain associated disturbances to the surrounding marine ecosystem have become apparent. Among the most notable is the

deposition of particulate organic matter underneath cages at salmon aquaculture sites (Karakassis et al., 2000; Carroll et al., 2003; Sarà et al., 2004; Corner et al., 2006; Jusup et al., 2009). This complex mixture of sediment, also known as flocculent matter, is mainly comprised of organic material from decomposing fish-food pellets and fish faeces (Ye et al., 1991; Strain and Hargrave, 2005; Salvo et al., 2015), and has been known to drive benthic community structure changes (Pearson and Rosenberg, 1978) and physiochemical modifications to existing seafloor environments (Shaanning, 1994; Hamoutene, 2014). During production, flocculent matter settles onto the seafloor and stimulates bacterial activity, causing a local decrease in oxygen levels. Where hypoxic and anoxic conditions develop, sulfides and methane may also accumulate (Hargrave et al., 2008).

Monitoring of organic waste deposition from aquaculture activity is typically conducted by obtaining sediment via grab or core sampling. Changes within the sediment can then be measured via redox potential, sediment sulphide concentration and/or faunal benthic community shifts to determine if there have been any potential environmental impacts from farming activity (Hargrave et al., 2008). These methods have drawbacks; for instance, they become less effective at measuring more subtle impacts as the distance from cages increases (Carroll et al., 2003), leading to the need for more sensitive methods. In addition, the duration of sample conservation and repeatability have been highlighted as significant methodological concerns.

Though these methods have been found to be useful for measuring the impact of organic enrichment (OE) at soft-bottom finfish sites, they are impractical and challenging where hard-bottom substrates are dominant, such as at farms found along the NL coast, especially over deep water (Hamoutene et al., 2013). Because grab sampling success is limited and unreliable in NL, monitoring of OE is conducted by obtaining video recordings of the

benthic environment in accordance with DFO standard monitoring practices (DFO, 2012, 2015). Drop-camera monitoring along the south coast of NL uses Opportunistic Polychaete Complexes (OPCs) and white microbial mats associated with flocculent matter deposition as visual indicators for OE (Hamoutene et al., 2014, 2015). Although video footage allows the reporting of general conditions at the seafloor (i.e. presence or absence of OE indicators), it may produce limited information on species abundance, especially at lower taxonomic levels and within sediments (Crawford et al., 2001). The OE indicators used in Canadian regulation are also sensitive to environmental conditions such as temperature (Salvo et al., 2017) and sulphide levels (Hamoutene, 2014). Barren stations with no observable species can also indicate high levels of OE during routine monitoring (DFO, 2015; Hamoutene et al., 2015).

3.1.2 Potential use of bacterial biomarkers in benthic monitoring

As an alternative to current monitoring methods, researchers have explored the use of high throughput sequencing techniques to characterize bacterial communities from aquaculture site sediments. Microorganisms play important roles in the breakdown of organic matter (Panikov, 1995) and are likely to be sensitive to environmental changes caused by organic loading, making them good candidates in monitoring efforts (Nogales et al., 2011; Wan et al., 2017). Bacterial community analysis through gene sequencing has been shown to be a cost-effective tool to monitor OE at farms (Keeley et al., 2018).

To perform bacterial community analysis through gene sequencing, sediment samples must first be obtained from grab or core contents; subsamples can be collected using swabs (e.g. Verhoeven et al., 2016, 2018) or placed directly into sterile tubes. DNA is then extracted from sediment subsamples and a fragment of the 16S rRNA gene, which is

frequently used as a taxonomic marker in bacteria (Janda and Abbott, 2007), is sequenced. From each sample, a series of gene sequences, which can be further analyzed using bioinformatic methods, is obtained. Typically, individual gene sequences are compared to a reference database (e.g. SILVA, Quast et al., 2013) and identified to the lowest possible taxonomic level, to produce a bacterial community profile for each sample. Bacterial communities can be compared across samples using statistical clustering approaches and grouped according to similarity in taxonomic composition. Samples from aquaculture sites examined in this manner have shown that community composition differs according to distance from cage and according to time in a production and fallow cycle (Verhoeven et al., 2018). Bacterial communities in sediments around aquaculture sites in NL group into four clusters (Verhoeven et al., 2018) hereafter classified as “High Impact”, “Recently Disturbed”, “Intermediate Impact” and “Low Impact”. Category names are based on several variables such as site status (production, fallow or reference), distance from cage edge, sediment organic matter content, and time of sampling in relation to production or fallow cycle (Verhoeven et al., 2018).

Bacterial sequence data has also been used to examine whether particular taxa might be biomarkers of organically enriched sediment at aquaculture sites. Dowle et al. (2015) found a high abundance of *Desulfobacterales* around cages and a high abundance of *Gammaproteobacteria* at sites unaffected by aquaculture activity in New Zealand. In contrast, Kawahara et al. (2009) found Betaproteobacteria in high abundance at impacted sites in Japan, but also measured high abundances of *Gammaproteobacteria* at less impacted sites. Verhoeven et al. (2016) found that *Spirochaeta*, *Prolixobacter* and *Marinifilum* were relatively abundant in samples obtained near cage edge at a 3-month fallow site in NL, and in a larger study, "High Impact" stations showed increases in

Bacteroidetes, Firmicutes and Spirochaetes relative to lower impact sites (Verhoeven et al., 2018). *Spirochaeta* were also strongly linked to OE in a New Zealand study (Keeley et al., 2018). Moreover, targeted sampling of bacterial mats at a NL site in fallow indicated that they were dominated by *Spirochaeta*, *Prevotella*, *Meniscus* and *Odoribacter* rather than *Beggiatoa* which is known to be typically present at aquaculture sites (Verhoeven et al., 2016).

While research suggests that bacterial community composition or bacterial biomarkers could be useful for assessing impacts of OE, more research is needed to relate sedimentary bacteria to the biological processes occurring during aquaculture production and fallowing. Relationships between bacterial communities and abiotic features of flocculent matter should also be characterized to establish the conditions under which particular bacterial taxa or assemblages can be detected. Establishing any linkages between bacterial communities and currently-used visual indicators of OE (OPCs/bacterial mats) would also be valuable as they could help to properly interpret environmental conditions associated with visual indicator presence.

3.1.3 Spatial and temporal patterns

Organic waste generated by intense fish farming disperses along a spatial gradient, decreasing in concentration as distance from cage edge increases (Ye et al., 1991; Carroll et al., 2003), but potentially reaching distances of over 300 m (Yokoyama et al., 2006). However, most deposition is concentrated near and around cages and associated effects on benthic communities appear most evident within < 50 m from cages (DFO, 2003). OE at aquaculture sites has been linked to changes in macrofaunal assemblages near cages

(Cathalot et al., 2012) and along gradients (Karakassis et al., 2000) as well as losses in macrofaunal community diversity (Brooks et al., 2003).

Bacterial community responses are similar to those of known macrofaunal indicators of environmental stress from aquaculture activity (Stoeck et al., 2018), which supports the concept of using bacteria as bioindicators in aquaculture environmental impact assessment (Aylagas et al., 2016). Spatial studies of bacterial communities at aquaculture sites have shown similar reaction to benthic fauna, with community shifts occurring more prominently near cages where OE is highest (Tamminen et al., 2011; Luna et al., 2013). Bacterial community successions have also been noted over distance gradients at aquaculture sites (Kawahara et al., 2009).

Temporal changes in bacterial communities at aquaculture sites have received little attention, especially during fallowing periods. Recovery times for complete (geochemical and biological) remediation varies according to studies and parameter measured, and could take anywhere from 7-14 weeks (Brooks et al., 2003), 6 months (Brooks et al., 2003), or longer than 5 years (Brooks et al., 2004; Keeley et al., 2014). For benthic faunal communities, a full recovery could take at least a few months once production ends (Moverly, 1995; Keeley et al., 2014), and at some NL locations, bacterial communities showed little to no recovery after 35 months of fallowing (Verhoeven et al., 2018). These studies highlight the inconstancies in recovery times at aquaculture sites, which are most likely caused by differences in site location, age, farming intensity, ocean currents and bathymetric characteristics.

3.1.4 Objectives

In this study, we examine how bacterial communities in sediments (organically enriched and natural) from NL aquaculture sites experiencing organic enrichment (production) and remediation (fallowing) relate to the abiotic sediment characteristics investigated in Chapter 2, and to the presence or absence of bacterial mats. This work focuses on a subset of the samples analyzed by Verhoeven et al. (2018), who determined categories of bacterial indicators after high-throughput sequencing of the 16s rRNA gene in DNA isolated from sediments. We hypothesize that: 1) bacterial mats will be associated with certain bacterial communities; and 2) differences in bacterial communities will be associated with changes in sediment abiotic characteristics. Results of this study should provide us with a better understanding of bacterial community composition and its association with bacterial mats in response to OE during production and fallow periods at NL salmonid farms and other hard-bottom aquaculture sites.

3.2 Materials and methods

3.2.1 Study sites

Sampling was conducted in 2016 and 2017 at two aquaculture sites along the southern coast of NL, where substrates are predominantly rocky with patches of sediment; the exact location of those sites cannot be disclosed at the request of the aquaculture industry. The two study sites were located near Hermitage Bay and Belle Bay in the Coast of Bays region. Both sites experienced multiple rounds of production, with the production site starting its latest cycle in May 2016 after an 11-month fallow period. The fallow site was most recently stocked in 2012, harvested in 2014 and subsequently transitioned into fallow. In 2017, samples were collected from an additional site in Belle Bay and used as a baseline. This

site was ideal for reference sampling as it was located >500 m away from aquaculture activity and had sediment buildup. For complete details on the sites used in this study, please refer to Chapter 2 Section 2.2.1 Study Sites.

3.2.2 Sampling procedure

Areas with a buildup of sediment or flocculent matter were identified using underwater video monitoring in accordance to standard monitoring practices (DFO, 2012). This was performed prior to sampling to locate potential grab sampling stations along transects starting from cage edge (0 m) and extending up to 160 m away from the cage array. Details regarding video monitoring, station selection and grab sampling can be found in *Chapter 2, Section 2.2.2 Data Collection*. Once stations were selected, collection of sediment was performed using an Ekman grab (6 x 6 x 10 inches). From each successful grab, we first collected up to three bacterial samples using sterile polyester swabs (Starplex Scientific Inc.): three swabs in the first year of sampling, and one swab inserted successively at 3 sublocations at the surface of the grab the second year. Swabs were stored in a transport medium (Starswab Multitrans System, Starplex Scientific Inc.) and kept on ice until transported to shore, where all samples were frozen at -20°C. Subsamples of grab contents were transferred to 20 ml scintillation vials pre-burned at 450˚C for 4 h for trace element analysis, and to 50 ml plastic centrifuge tubes for % organic matter and CHN analysis; all subsamples were stored on ice. Sediment and microbial samples were then brought to a laboratory at Memorial University and stored at -80°C. For details on bacterial swabs collected during sampling, please refer to *Supplementary Table D.1*. For additional details on sediment collection and related analyses please refer to *Chapter 2 Section 2.2 Materials and Methods*.

3.2.3 Analytical procedures

3.2.3.1 Sample processing and taxonomic profiling

To determine bacterial community compositions after collection, we performed nucleic acid extractions using AllPrep PowerViral DNA/RNA Kit (Qiagen) and quantification using a NanoDrop ND-1000 (Thermo Fisher Scientific). All extractions were performed at Memorial University within a few months of sampling. 10 ng/µl of each extract sample was loaded into PCR tube wells, packaged for shipping and sent to the Integrated Microbiome Resource (IMR) at the Centre for Comparative Genomics and Evolutionary Bioinformatics (Dalhousie University, Halifax, Canada). Bacterial 16S rRNA gene sequencing was performed via Illumina MiSeq 300 bp paired-end sequencing of the 16S V6-V8 region and amplified using universal bacterial primers B969F and B1406R (Comeau et al., 2017). Once the sequencing results were obtained, processing and bacterial community analyses were performed.

The bioinformatic analysis of bacterial communities was described in Verhoeven et al. (2018) and is not detailed in full here. The analysis considered a larger number of stations than those presented in this study, and resulted in the identification of four clusters of stations, differing significantly in bacterial community composition. A subsequent pairwise comparative analysis identified bacterial genera that differed significantly in abundance between pairs and were referred to as biomarkers (Verhoeven et al., 2018). Here, we assigned the corresponding, pre-determined community cluster identity (from Verhoeven et al., 2018) to each of the samples under consideration. As co-author of Verhoeven et al. (2018), my participation included the collection and processing of samples (larger dataset including samples used for this study), nucleic acid extractions, NanoDrop quantification and writing part of the manuscript.

3.2.4 Statistical analyses

3.2.4.1 Relationship between bacterial communities and mats

To explore the relationship between bacterial communities and the presence of bacterial mats, we combined bacterial mat presence/absence data extracted from videos obtained at each station (see Section 2.2.2.1) with the corresponding community cluster identity (from Verhoeven et al., 2018), for all stations and sampling years. Data were sorted by cluster group and tabulated. Presence/absence data was also applied to stations on site map figures for visualization and to investigate community shifts over time. To determine which operational taxonomic units (OTUs) were significantly associated with stations in the mat presenting group, we chose to utilize the ANOVA-Like Differential Expression (ALDEx2) R package on our high-throughput sequencing data (Fernandes et al., 2013, 2014). If an OTU had an negative effect size of (≤-1), it was considered significantly more associated with stations having mats.

3.2.4.2 Relationship between bacterial communities and sediment characteristics

To explore how sediment characteristics may relate to aquaculture and bacterial community cluster identity, we used a multivariate approach within Primer 7 and PERMANOVA+ add on (Clarke and Gorley, 2015; Anderson, 2017) on a reduced number of variables: we sub-selected sediment characteristic variables based on their correlation (see Chapter 2). First, we combined selected data in a single database for each sampling station including: 1) selected sediment variables [%TOC, TOC/TN Ratio, %OM, Zn, Cd, Pb, Cr (log) and Cu (log)]; 2) factors such as site status, year of sampling, distance from cage, and 3) bacterial community cluster identity based on Verhoeven et al. (2018). Second, sediment data were normalized and similarity between stations assessed using Euclidian

distance. Then, we ran a Principal Component Analysis (PCA) and a PERMANOVA to test whether abiotic sediment characteristics differed significantly according to site (i.e. production, fallow or reference) or assigned bacterial community cluster identity. For the PERMANOVA, we tested two factors (site and bacterial community cluster identity) that were set as fixed and the model was ran using 9,999 permutations and type 1 sums of squares (sequential, unbalanced design). The contrast option was used in the model to test for differences between grouped aquaculture sites (i.e. fallow and production) and reference stations. When significant, pairwise comparisons were also completed. As bacterial community cluster identity was significant, we also ran a Kruskal-Wallis *H* test in Sigma Plot 13.0 to determine whether there were significant differences in concentrations for each sediment parameter between community clusters. A post-hoc test using the Dunn's pairwise multiple comparison procedure was also done to test for statistically significant differences between community cluster pairs.

3.3 Results

3.3.1 Bacterial community cluster distribution

71 swabs (from 39 stations) were successful in 16S rRNA gene high-throughput sequencing; one swab sample for a 2017 production station (0 m from cage edge) could not be assigned to a cluster due to low read counts and was classified as not applicable (N/A). Nucleic acid extraction was not performed for one 2016 fallow station sampled at 0 m from cage edge (screened out prior to nucleic acid extractions) and was also classified as N/A (Refer to Appendix D for station details). In all cases where replicate swab samples were collected from a grab, we found the same community cluster across those replicates. The four bacterial community clusters from Verhoeven et al. (2018) were represented among

the swab samples obtained from production, fallow and reference stations in 2016 and 2017.

Based on the subset of bacteria samples considered in this study (relative to the larger sample set in Verhoeven et al., 2018), the High Impact cluster was characteristic of stations that were mostly in close vicinity to cages (0-20 m, 85% of stations), at production (*n*=12) and in fallow (*n*=8) (Table 3.1). In contrast, the Low Impact cluster was only found in fallow stations at 0-160 m from cages, with 76% of samples being found at ≥40 m from cages. The two reference samples also grouped with this cluster. The Recently Disturbed cluster contained samples exclusively from the production site ranging 0-40 m from cages (*n*=13), while the Intermediate Impact cluster had mostly samples from the fallow site (*n*=10, 91% of samples) at 0-80 m from cages. One sample from the production site, sampled at 40 m from cage edge, also grouped with the Intermediate Impact cluster. When comparing sites over time (Figs. 3.1-3.4), the production site remained dominated by High Impact and Recently Disturbed clusters between 2016-2017. In contrast, the fallow site was found to have mostly Low Impact and with few Intermediate Impact communities in 2016. 2017 sampling revealed shifts from Low Impact to Intermediate and High Impact communities especially along the 3S transect. A fallow station along the 8W transect at 0 m from cage edge deviated from this trend as it shifted from a High Impact to an Intermediate Impact community between 2016-2017.

Table 3.1. Summary of bacterial swab samples and their associated bacterial community cluster from Verhoeven et al. (2018) where *n*= number of bacterial swab samples extracted from grabs at stations in 2016 and 2017.

	High Impact	**Recently Disturbed**	**Intermediate Impact**	**Low Impact**
Production Samples (*n=26*)	**12**	**13**	**1**	**-**
2016	7	9	-	-
2017	5	4	1	-
Distances from cage				
0 m	6	3	-	-
5 m	-	3	-	-
20 m	5	3	-	-
40 m	-	2	1	-
80 m	-	-	-	-
120 m	-	-	-	-
160 m	-	-	-	-
Center cage	1	2	-	-
Fallow Samples (*n=43*)	**8**	**-**	**10**	**25**
2016	3	-	4	17
2017	5	-	6	8
Distances from cage				
0 m	5	-	3	1
20 m	-	-	1	5
40 m	1	-	5	4
80 m	-	-	1	5
120 m	2	-	-	5
160 m	-	-	-	5
Reference *(n=2)*				
2017	-	-	-	2
Total samples *(n=71)*	**20**	**13**	**11**	**27**
2016	10	9	4	17
2017	10	4	7	10

3.3.2 Relationship between bacterial community clusters and mats

We investigated the relationships between each community cluster and the presence of bacterial mats, based on observations of extracted images from video monitoring at each station, for both sampling years (Table 3.2 and Figs. 3.1-3.4). Bacterial mats were associated with all four clusters, and were present at the majority of stations (29 out of 39 surveyed). N/A samples that were not analyzed for community clusters also had mats present at their stations. An absence of mats was observed mainly at stations characterized by the Low Impact cluster (9 out of 14 stations), with one station grouped within the High Impact cluster showing no evident bacterial mat. Upon visual inspection, this station differed from others, as there appeared to be less sediment buildup and some native macrofauna (sea stars) were present. All stations that were characterized by Recently Disturbed or Intermediate Impact clusters were identified as having mats.

In general, mats found at production and fallow stations differed visually; mats at production stations were typically less dense and sparser within a quadrat (Fig. 3.5A) while at fallow stations they appeared denser (Fig. 3.5B) and appeared to cover the majority of the quadrat**.** However, bacterial mats at production stations in 2017 increased in density (Fig. 3.5C) compared to the previous sampling year while at fallow stations they remained unchanged visually across years (Fig. 3.5D). When comparing among bacterial mats found associated with different bacterial community clusters, there were no obvious trends. At production stations, mats associated with High Impact and Recently Disturbed communities were typically whiter, more defined, less dense and accompanied with OPC. Sediment below mats was also darker in appearance compared to other clusters. At fallow stations, mats were visually similar across High Impact, Intermediate Impact and Low

Impact stations (as seen in Fig. 3.5). Low Impact stations without mats had a large buildup of ‘natural’ sediment that resembled reference station images (Fig. 3.6).

Results from ALDEx2 analysis revealed that 9 OTUs, respectively identified to the lowest possible taxonomic level as belonging to the class Deltaproteobacteria, the genus *Spirochaeta,* the order Cloacimonadales and the order Bacteroidales (n=6) were significantly associated with mat presence.

Table 3.2. Summary of stations found with and without bacterial mats, sorted by bacterial community cluster. *n*= number of stations.

Clusters		High Impact	Recently Disturbed	Intermediate Impact	Low Impact	N/A
Presence of Mats	**(*n*)**	**9**	**7**	**8**	**5**	**2**
	Production Samples (*n*)	**5**	**7**	**1**	**-**	**1**
	2016	2	3	-	-	-
	2017	3	4	1	-	1
	Fallow Samples (*n*)	**4**	**-**	**7**	**5**	**1**
	2016	1	-	2	4	1
	2017	3	-	5	1	-
	Distances from cage					
	0 m	5	1	3	1	2
	5 m	-	1	-	-	-
	20 m	1	1	1	2	-
	40 m	1	2	3	-	-
	80 m	-	-	1	1	-
	120 m	1	-	-	1	-
	160 m	-	-	-	-	-
	Center cage	1	2	-	-	-
	Reference (>500 m)	-	-	-	-	-
Absence of Mats	**(*n*)**	**1**	**-**	**-**	**9**	**-**
	Production Samples (*n*)	**1**	**-**	**-**	**-**	**-**
	2016	1	-	-	-	-
	2017	-	-	-	-	-
	Fallow Samples (*n*)	**-**	**-**	**-**	**7**	**-**
	2016	-	-	-	3	-
	2017	-	-	-	4	-
	Distances from cage					
	0 m	-	-	-	-	-
	5 m	-	-	-	-	-
	20 m	1	-	-	-	-
	40 m	-	-	-	2	-
	80 m	-	-	-	2	-
	120 m	-	-	-	1	-
	160 m	-	-	-	2	-
	Center cage	-	-	-	-	-
	Reference (>500 m)	-	-	-	2	-
Total Stations *(n=41)*		**10**	**7**	**8**	**14**	**2**

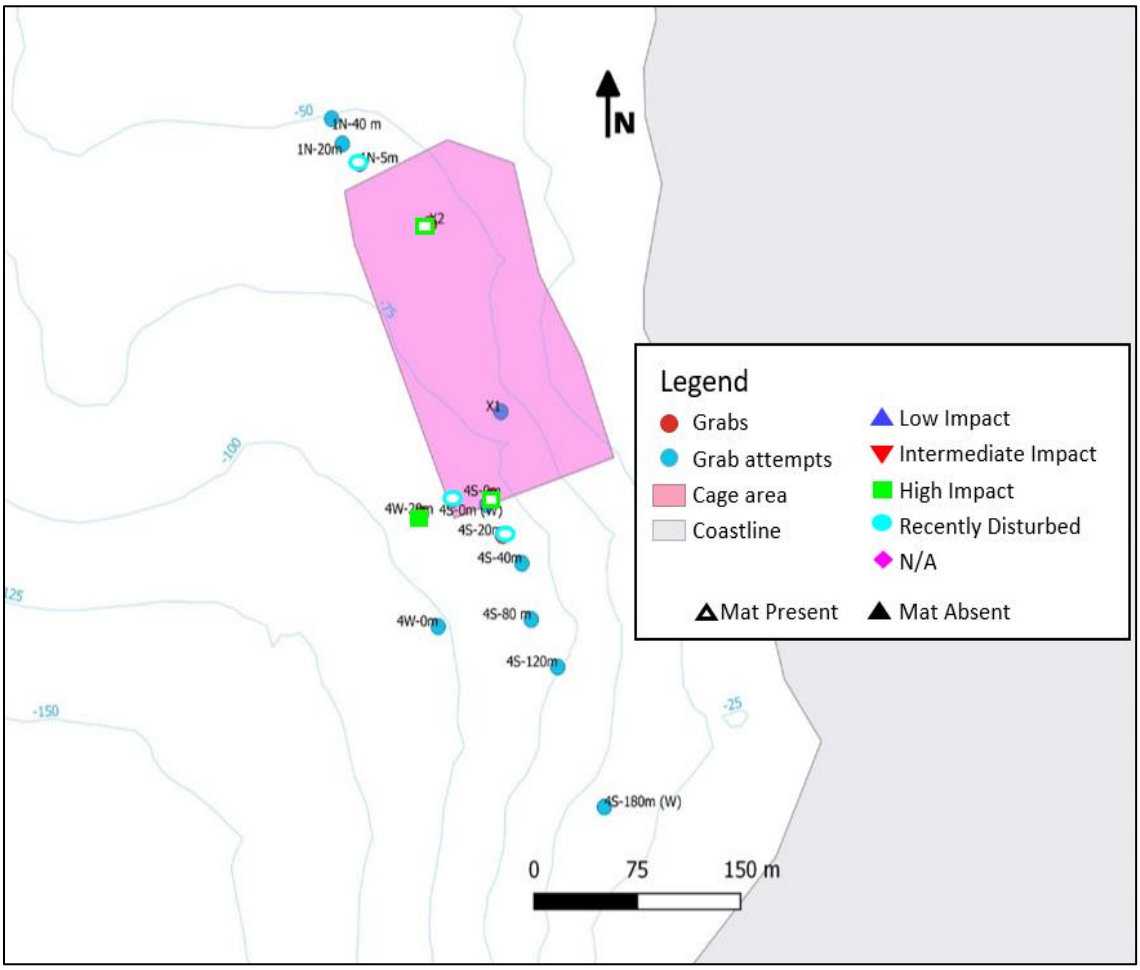

Figure 3.1. Map of production site showing grab sampling stations from 2016 labelled according to the corresponding bacterial community cluster (Low Impact, Intermediate Impact, High Impact, Recently Disturbed and N/A) and presence/absence of bacterial mats.

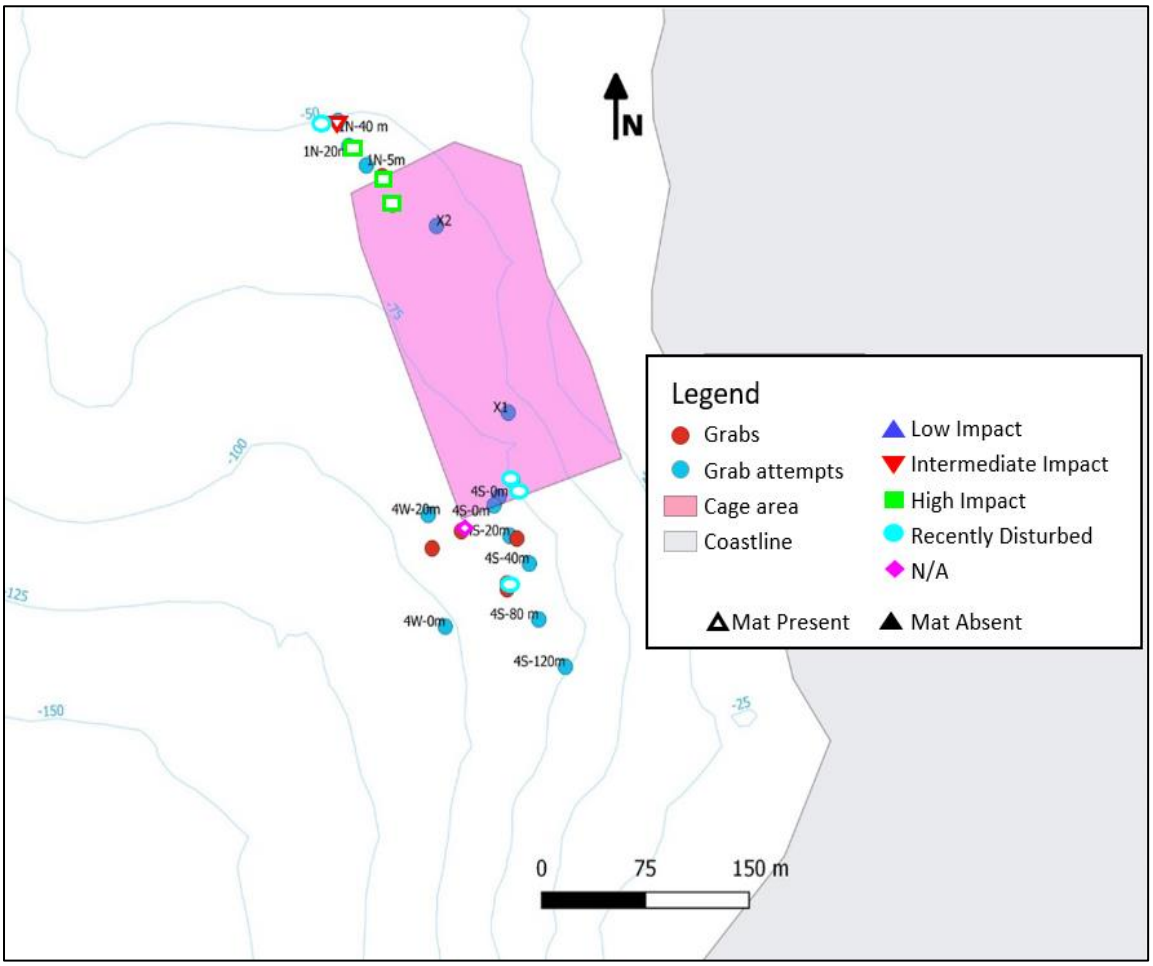

Figure 3.2. Map of production site showing grab sampling stations from 2017 labelled according to the corresponding bacterial community cluster (Low impact, Intermediate Impact, High Impact, Recently Disturbed and N/A) and presence/absence of bacterial mats.

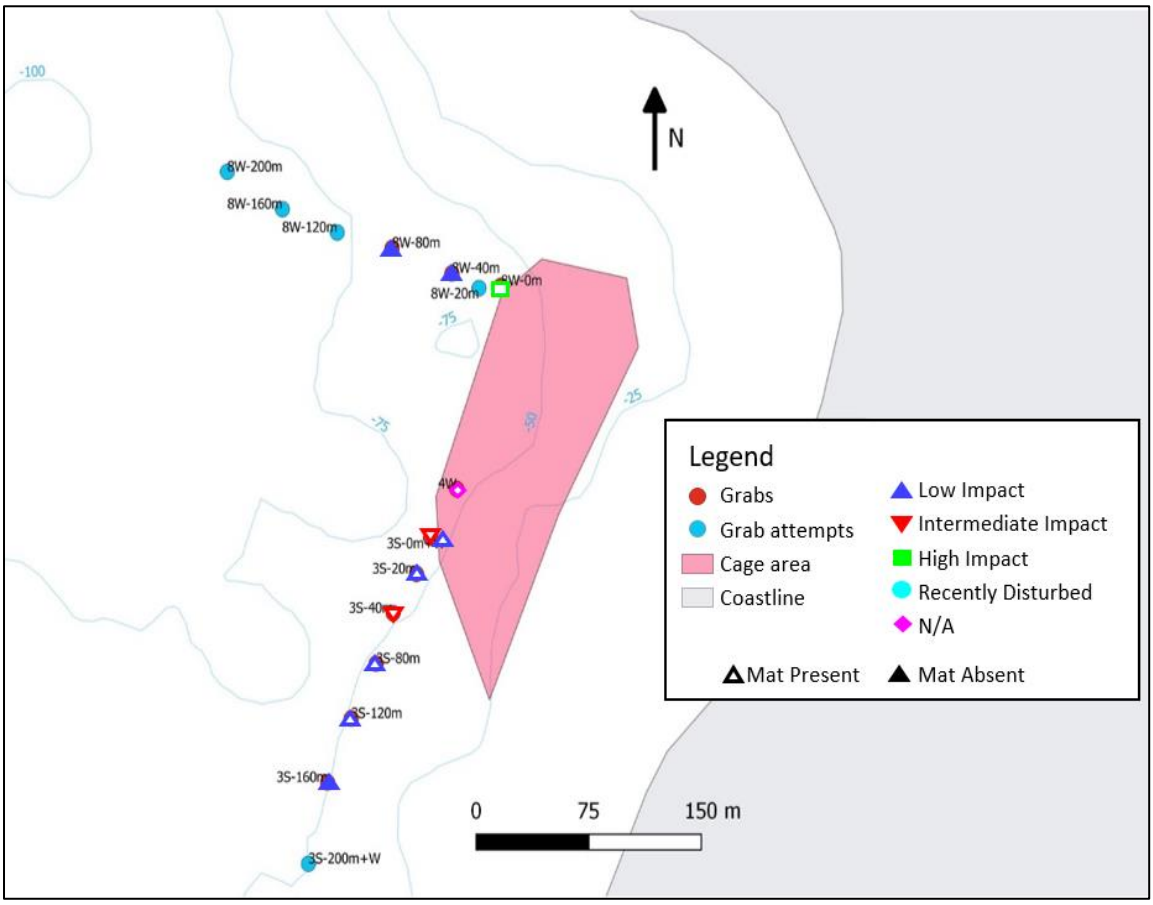

Figure 3.3. Map of Fallow site showing grab sampling stations from 2016 labelled according to the corresponding bacterial community cluster (Low Impact, Intermediate Impact, High Impact, Recently Disturbed and N/A) and presence/absence of bacterial mats.

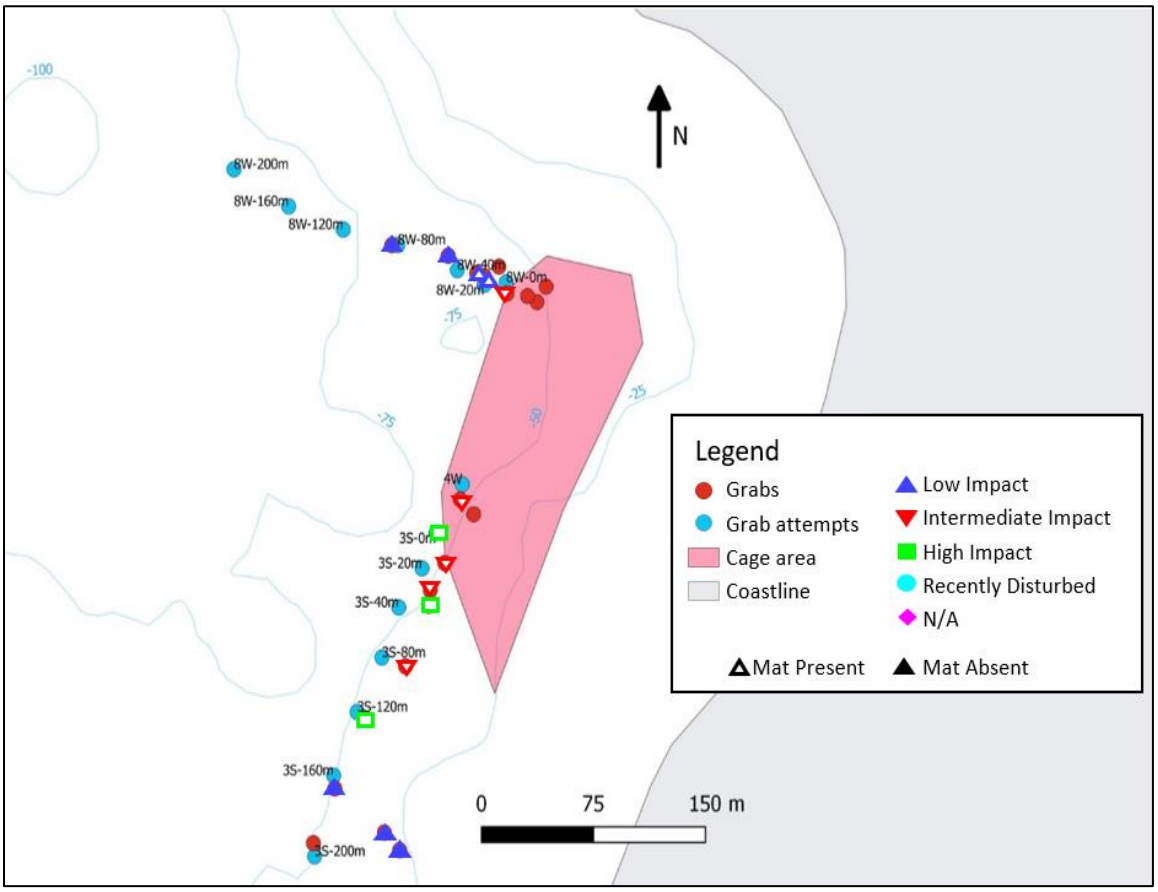

Figure 3.4. Map of Fallow site showing grab sampling stations from 2017 labelled according to the corresponding bacterial community cluster (Low Impact, Intermediate Impact, High Impact, Recently Disturbed and N/A) and presence/absence of bacterial mats.

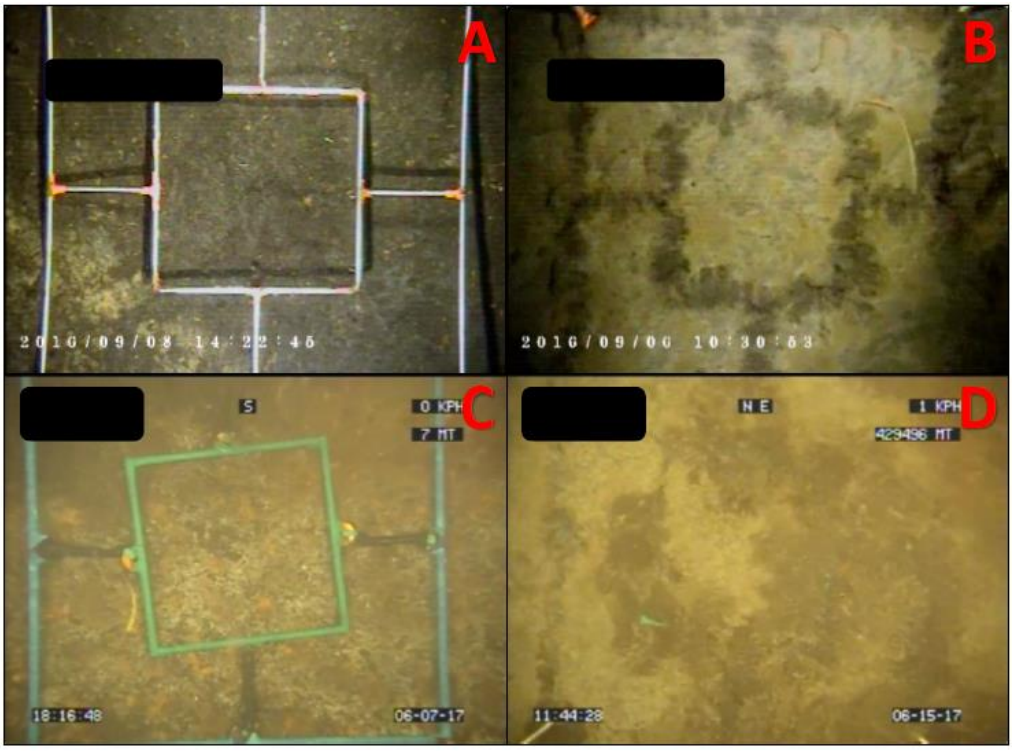

Figure 3.5. Extracted seafloor images at stations from video monitoring illustrating general visual differences between bacterial mats at A) production 2016 stations, B) fallow 2016 stations, C) production 2017 stations (with OPC clusters in pink) and D) fallow 2017 stations.

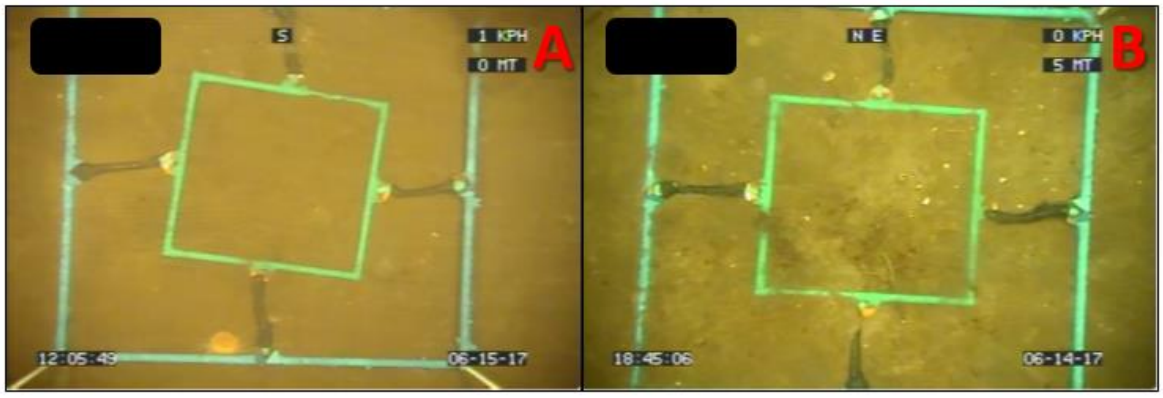

Figure 3.6. Extracted seafloor images at stations from video monitoring illustrating general visual similarities between A) Low Impact stations without mats from the fallow site and B) Reference stations.

3.3.3 Relationships between communities and sediment characteristics

The principal components analysis (PCA) run on selected sediment parameters revealed differences among community clusters, particularly between High Impact and Low Impact stations (Fig. 3.7). The first PCA axis (PC1) explained 50.4% of the total variation while the second axis (PC2) explained 31.7%, for a total of 82.6%. The vectors for %TOC and TOC/TN were oriented in an opposite direction to the Cr(log) and Pb vectors. Along PC1, High Impact and Recently Disturbed stations tended to be separated from other stations, with %TOC and TOC/TN, and to a lesser extent Zn, %OM, Cu(log) and Cd vectors being more in alignment with the High Impact and Recently Disturbed stations than with the others. Along PC2, stations tended to show a greater variability. The Low Impact (including reference stations) and Intermediate Impact stations were grouped together and opposite to the %TOC, TOC/TN, %OM, Zn, Cu(log) and Cd vectors, but more in alignment with Pb (Fig. 3.7). High Impact and Recently Disturbed stations showed a high variability in sediment characteristics.

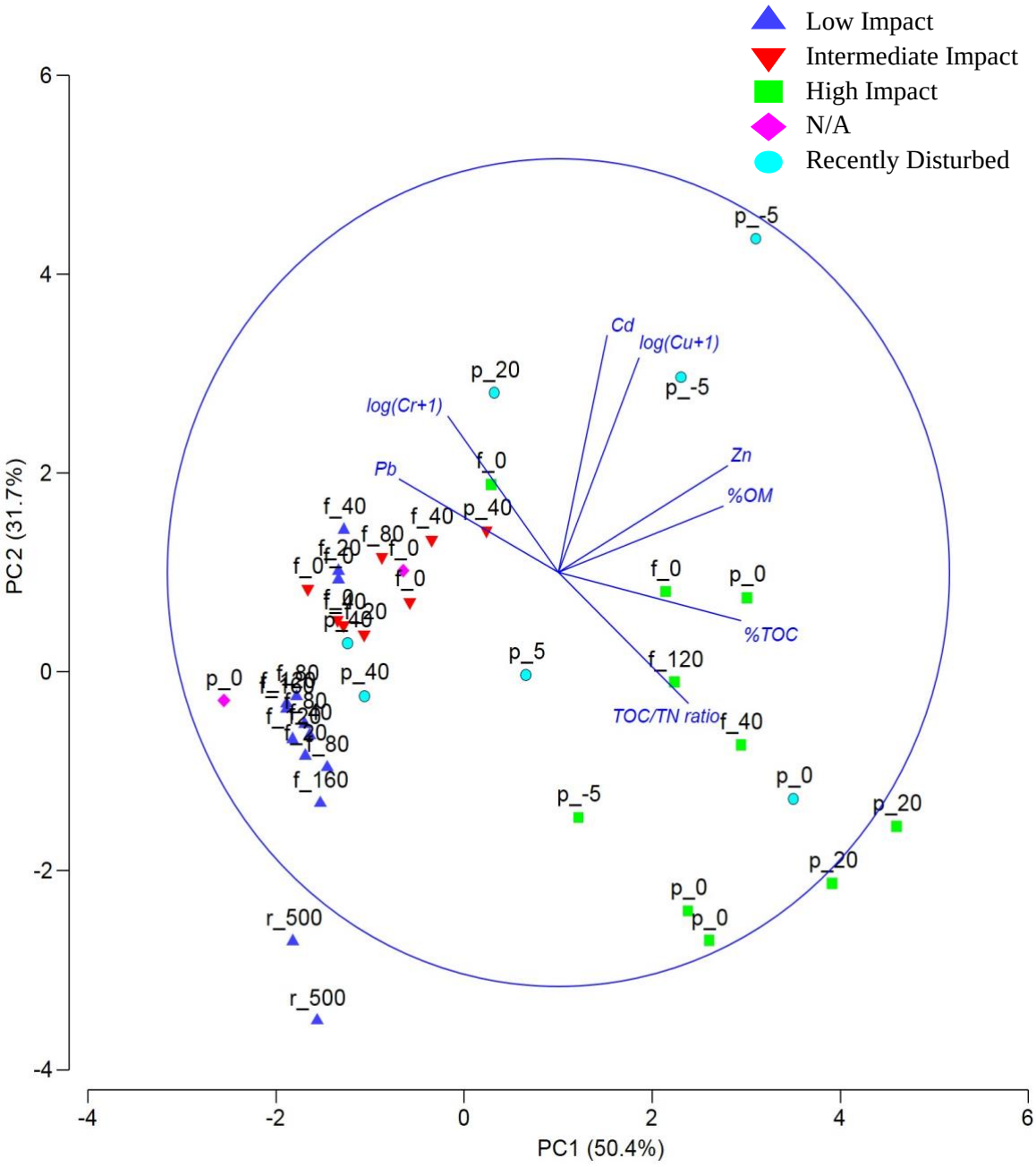

Figure 3.7. Principal components analysis of sediment abiotic characteristics, with samples color-coded by bacterial community cluster. Correlation vectors for each variable (sediment characteristic) are superimposed. Labels represent site status (p=production, f=fallow and r= reference) followed by distance from cage edge in meters. Distances of -5 m are at centre of cage array. N/A= stations that were not analyzed for bacterial community composition.

PERMANOVA results showed a significant effect of site on abiotic sediment characteristics (Table 3.3), when comparing all sites and when comparing aquaculture sites (production and fallow sites combined) to reference stations. Pairwise tests further indicated that fallow, production and reference sites differed significantly from each other (p<0.001) with Euclidian distances being greatest between fallow and reference, and between production and reference sites (Table 3.4).

A significant effect of bacterial community cluster identity on sediment characteristics was also found using PERMANOVA (Table 3.3). Pairwise testing indicated significant differences between each pair of community clusters, except for comparisons with N/A (n=2), with the biggest difference being between Low Impact and High Impact stations (Euclidian distance = 4.9048***) and between Recently Disturbed and Low Impact stations (Euclidian distance =4.4864**) (Table 3.5). Interestingly, the greatest within-group variation was documented for the Recently Disturbed (4.4115) and High Impact (3.2323) clusters, confirming PCA observations.

Table 3.3. PERMANOVA results on selected sediment characteristics to test differences between site status and bacterial community cluster. df= degrees of freedom; SS= sum of squares; Pseudo-F= F value by permutation; Sq.root= square root. *P*-values are based on 9999 permutations with significant *p*-values bolded.

Source	df	SS	Pseudo-F	*P*(perm)	Sq.root
Site (Fallow, Production and Reference)	2	88.35	11.445	**0.0001**	1.9426
Reference vs. Aquaculture Site	1	32.493	7.9678	**0.0001**	2.7328
Bacterial Community Cluster	4	100.42	6.504	**0.0001**	1.8643
Residuals	34	131.23			1.9646
Total	40	320			

Table 3.4. Pairwise tests on site status to show average distance between/within groups (italic) on the PERMANOVA results on selected sediment characteristics. Significant values are denoted as: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. *n*= number of stations.

	Fallow	Production	Reference
Fallow (*n*=24)	*2.2315*		
Production (*n*=15)	4.1437***	*4.4601*	
Reference (*n*=2)	4.2713***	5.5456**	*1.0187*

Table 3.5. Pairwise test for bacterial community clusters to show average distance between/within groups. Significant values are denoted as: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

	Low Impact	Intermediate Impact	High Impact	N/A	Recently disturbed
Low Impact	*1.9885*				
Intermediate Impact	2.2489*	*1.3964*			
High Impact	4.9048***	4.3737**	*3.2323*		
N/A	2.1782	1.825	4.905*	*2.5965*	
Recently disturbed	4.4864**	3.5625*	4.4617*	4.2421	*4.4115*

Averages of sediment concentrations for each bacterial community cluster identity (Table 3.6) confirmed PCA trends. Stations with High Impact and Low Impact community clusters were the most divergent in terms of concentrations. %TOC, TOC/TN and %OM were highest at the High Impact stations but were low at Intermediate and Low Impact stations. Zn was also noticeably higher at Recently Disturbed (528.43 ± 265.73 ppm) and High Impact stations (512.70 ± 123.7 ppm) compared to the Low Impact cluster (106.86 ± 52.53 ppm). In contrast, Pb was highest at Low Impact stations and lowest at High Impact stations, 22.87 ± 6.17 ppm and 8.24 ± 5.93 ppm, respectively. Less obvious trends were evident for Cr(log), Cu(log) and Cd as differences in sediment concentrations were less apparent among clusters.

Kruskal-Wallis Tests were conducted to examine differences in abiotic sedimentary characteristics across community cluster groups (Table 3.7). Significant differences were found for all selected characters except Cd. Post-hoc Dunn's tests revealed significant differences between High Impact and Low Impact stations, where %TOC, %OM, Zn and Cu (log) had the strongest significance levels ($p<0.001$). Recently Disturbed and Low

Impact stations also showed significant differences for most characteristics (%TOC, Zn, Pb, Cr (log) and Cu (log)), while differences between High Impact and Intermediate Stations were found for the TOC/TN ratio and Pb. Recently Disturbed and Intermediate stations differed only in Pb concentrations. Dunn's post hoc comparisons could not be performed in some instances due to the low number of samples within certain groups (n=7, low and intermediate stations).

Table 3.6. Average sediment characteristic values at stations associated with each bacterial community cluster.

	High Impact (Avg. ± SD, *n*= 10)	**Recently Disturbed** (Avg. ± SD, *n*= 7)	**Intermediate Impact** (Avg. ± SD, *n*= 8)	**Low Impact** (Avg. ± SD, *n*= 14)
%TOC	28.96 ± 8.11	16.55 ± 13.10	7.83 ± 1.53	4.41 ± 1.92
TOC/TN	10.75 ± 2.57	7.01 ± 2.53	6.59 ± 0.74	7.46 ± 0.96
%OM	39.22 ± 17.84	32.06 ± 23.60	18.88 ± 4.64	11.56 ± 6.06
Zn	512.70 ± 123.7	528.43 ± 265.73	264.75 ± 85.22	106.86 ± 52.53
Cd	0.82 ± 0.44	1.08 ± 0.61	0.96 ± 0.17	0.58 ± 0.32
Pb	8.24 ± 5.93	12.09 ± 4.89	23.13 ± 3.38	22.87 ± 6.17
Cr(log)	1.13 ± 0.23	1.43 ± 0.25	1.46 ± 0.09	1.42 ± 0.16
Cu(log)	1.64 ± 0.13	1.88 ± 0.32	1.73 ± 0.13	1.46 ± 0.22
Stations (*n= 39*)	***HIGH IMPACT*** *Production (n= 6)* *Fallow (n= 4)* *Reference (n= 0)*	***RECENTLY DISTURBED*** *Production (n= 7)* *Fallow (n= 0)* *Reference (n= 0)*	***INTERMEDIATE IMPACT*** *Production (n= 1)* *Fallow (n= 7)* *Reference (n= 0)*	***LOW IMPACT*** *Production (n= 0)* *Fallow (n= 12)* *Reference (n= 2)*

Note: Element concentrations in ppm.

Table 3.7. Results of Kruskal-Wallis test and the post hoc Dunn's multiple comparisons test, showing significance of differences in sediment characteristics between community clusters. HI= High Impact, LI= Low Impact, INT= Intermediate Impact, RD= Recently Disturbed community clusters; df, degrees of freedom; *H*, *H* test value; ns, non-significant; *p*, significance level; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; --, test not performed due to the low number of samples in certain groups (< 8).

	Kruskal-Wallis test			Dunn's multiple comparisons test					
				HI-LI	HI-INT	HI-RD	RD-LI	RD-INT	INT-LI
	df	*H*	*p*	*p*	*p*	*p*	*p*	*p*	*p*
%TOC	3	26.653	<0.001	***	ns	--	*	--	ns
TOC/TN Ratio	3	15.235	0.002	**	**	ns	ns	--	--
%OM	3	14.883	0.002	***	ns	--	ns	--	--
Zn	3	28.607	<0.001	***	ns	--	***	--	ns
Cd	3	7.354	0.061	No statistically significant difference					
Pb	3	23.803	<0.001	***	*	ns	***	*	ns
Cr (log)	3	12.487	0.006	*	ns	--	*	--	ns
Cu (log)	3	15.769	0.001	**	ns	--	*	--	ns

3.4 Discussion

Previous studies have explored bacterial community composition within enriched sediments from predominantly hard-bottom aquaculture sites in NL (Verhoeven et al., 2016, 2018). Here, we expanded upon these studies by linking bacterial communities to drop-camera data (bacterial mat presence) and sediment characteristics (from Chapter 2) from NL aquaculture sites. This is the first study in the region to link abiotic characteristics of aquaculture enriched sediments and bacterial mat presence obtained through routine video monitoring with bacterial community composition.

While sediment grab sampling was difficult, we obtained 41 grab samples over the two-year study, from which 71 bacterial swab samples (some being replicates within a same grab) yielded 16S rRNA gene sequence data. Results from Chapter 2 indicated that OE resulting from aquaculture activities significantly altered substrates off the south coast of NL, with elevated concentrations of several measured parameters (OM in particular) in close proximity to cages, regardless of site status. Here, we found considerable overlap in bacterial communities from production and fallow sites, with bacterial community composition varying spatially and temporally at both sites. General trends indicated that the type of community showed a close association with the concentration of organic matter present, as observed previously (Verhoeven et al., 2018). Relationships between bacterial community clusters and bacterial mat appearance were also explored in the context of enrichment and remediation processes. General visual trends indicated a temporal pattern of mat growth starting during production periods and remaining stable over time during fallow periods.

3.4.1 Bacterial communities over space and time

Bacterial community distribution over space was not as clear-cut as expected; however, most communities at the production site were categorized as High Impact (*n*=6/15) and Recently Disturbed (*n*=7/15) whereas the fallow site contained mostly Low Impact (*n*=12/24) and Intermediate Impact (*n*=7/24) communities. High Impact and Recently Disturbed bacterial communities were present mostly near cages (≤ 40 m). Interestingly, the only Intermediate Impact community found at the production site was also at 40 m from cages, indicating a possible community transition at around this distance from cages. This is likely the result of the dispersal pattern of fish waste which in some areas has been shown to distribute along a gradient up to ~50 m from cages via currents (Lumb, 1989; Ye et al., 1991; Chou et al., 2002; Carroll et al., 2003). Spatial heterogeneity in bacterial communities at the production site could be from patchiness in OE due to fluctuations in direction and strength of currents (Hamoutene et al., 2015, 2016). Repeated cycles of production, as well as cage displacement up to 40 m from cages could also be contributing factors; however, an increase in sampling size on a larger spatial scale is needed to draw conclusions.

Community distributions showed no consistent change between 2016 and 2017 samplings. Production stations remained dominated by High Impact and Recently Disturbed communities between 5-13 months of production. At the fallow site, the presence of High Impact communities even after 4 years of remediation suggested that site was still experiencing enriched sediment conditions similar to production periods. Although the presence of Low Impact communities (as at the reference site) between 0 and 160 m from cage edge at the fallow site in 2016 could indicate signs of recovery, it is notable that these communities were accompanied by bacterial mats. Interestingly, shifts from Low Impact to Intermediate and High Impact communities were noted between 24-34 months of fallow,

especially along the 3S transect. This could not be readily related to changes in sediment conditions: findings from Chapter 2 indicate no significant change in sediment concentrations over a 10-month remediation period along the 3S transect (apart from Zn). Slow changes in sediment conditions have been confirmed in other studies (Neofitou et al., 2010) with recovery times ranging from weeks (Ritz et al., 1989), to months (Lumb, 1989; Brooks et al., 2003) and years (Karakassis et al., 1999; Wan Hussin et al., 2012; Keeley et al., 2014), according to various environmental factors such as number of previous production cycles, farm location and age, bathymetry, currents and adjacent ambient communities (Macleod et al., 2007); it is also likely that cold temperatures in NL lead to slow recovery. It is unclear why 55% of the stations that were sampled both years at the fallow site experienced shifts to higher impacted communities (all on the 3S transect), showing an opposite trend to typical fallow recovery dynamics, especially since we saw the opposite shift from a High Impact to an Intermediate Impact community at 0 m from cages along the 8W transect. Differences between years could have been due to the remobilization and transport of organically enriched sediments during storms. Additionally, apparent shifts can be due to our inability to revisit exactly the same location from year to year (due to GPS positioning error and grab sampling imprecision), especially considering the patchy nature of substrates in this region. Sampling over longer time spans and monitoring currents near the seafloor could help provide a better understanding of the factors behind the observed community shifts.

3.4.2 Relationships between bacterial communities and mats

By identifying the presence of bacterial mats through extracted video monitoring images, we were able to conclude (from Chapter 2) that mats were abundant at both

production and fallow sites as a result of OE. In this chapter, we elaborated on this by linking bacterial mat presence to community clusters. As mentioned in Chapter 2, most stations examined in this study (31/41) had bacterial mats, regardless of site status and year. Surprisingly, after incorporating community clusters to our dataset, all four community clusters were found in association with mats. Therefore, mat presence cannot be interpreted as necessarily signaling high impact levels, at least from the perspective of bacterial community composition.

There were no clear visual differences between mat presence and associated community clusters. Visual differences between mat presence and clusters may exist, but due to limitations in current video monitoring technology (i.e. lighting, depth, potential turbidity and dimensionality of seafloor), they may be difficult to discriminate. Interestingly, there were differences between sites over time, providing a snapshot of a potential temporal pattern for mat growth. At the beginning of production, mat coverage was much lower compared to the latter year of sampling, suggesting that mats spread over time and appear to become denser with increasing OE. Images from fallow stations indicated a high density of mats across sampling years, suggesting these mats most likely formed during production periods and remained stable over time. %OM, Zn, Cd and Cu (log) were significantly higher at sites with bacterial mats (Ch. 2), supporting other studies that link Zn and Cd with aquaculture activity (Sutherland et al., 2007). However, our results should be taken with caution, as those observations were made at only two sites experiencing different production status.

ALDEx2 results indicated that some bacteria classified within the Deltaproteobacteria, *Spirochaeta,* Cloacimonadales and the order Bacteroidales were significantly associated with mat presence. In agreement with Verhoeven et al. (2016), mats were not composed of

Beggiatoa sp. as once thought, and members from the genus *Spirochaeta* were identified in both studies. While the mat-forming genera *Prevotella*, *Meniscus* and *Odoribacter* were not identified in this study, it is notable that these genera fall within the order Bacteroidales (phylum Bacteroidetes), a taxon in which 9 OTUs (not classified at genus level) were identified. Discrepancies between studies could be linked to patchiness of benthic sediment from fish farming outputs, which has the potential to cause variation among grab replicates from the same station (Brooks and Mahnken, 2003).

3.4.3 Relationships between bacterial communities and sediment characteristics

In Chapter 2, we confirmed that hard-bottom aquaculture sites off the coast of NL are affected by organic loading from farming by analyzing station-specific sediment parameters over time and space (%TOC, TOC/TN, %OM, Cr, Cu, Zn, Cd and Pb). While OE at aquaculture sites has been extensively studied (Carroll et al., 2003; Sarà et al., 2004; Anderson et al., 2005; Wang et al., 2012; Cranford et al., 2017; Hamoutene et al., 2018), relationships between bacterial communities and sediment characteristics are not well defined.

In this study we found that sediment parameters influenced differences among cluster type as confirmed by PCA, PERMANOVA and Kruskal-Wallis analyses. Differences among clusters were most apparent between High Impact and Low Impact stations through pairwise tests (Euclidian distance = 4.9048***) and Kruskal-Wallis post hoc tests where %TOC, %OM, Zn and Pb concentrations differed the most among this pair. Moreover, biomarker analyses performed in Verhoeven et al. (2018) indicated that High Impact clusters were more associated with bacterial genera from phyla Bacteroidetes, Firmicutes and Spirochaetes. These phyla are dominated by sulfate-reducing anaerobic bacteria,

indicating that High Impact communities are experiencing oxygen-depleted conditions that are typical of organic matter rich substrates. Species of Bacteroidetes, Firmicutes and Spirochaetes have also been discovered in organically enriched sludge water (Qiu et al., 2013; Miyazaki et al., 2014) and hypoxic sediments from meromictic lakes (Watanabe et al., 2015). Members from the Bacteroidetes and Spirochaetes were also identified to have an significant association with mats in this study.

High Impact and Recently Disturbed stations had a high degree of variation in sediment parameters and were more associated with %TOC and TOC/TN, and to a lesser extent Zn, OM and Cu(log) according to the PCA. Although Cd vectors were more in alignment with the High Impact and Recently Disturbed stations than with the others, interestingly, Cd concentrations did not differ significantly between clusters. This suggests that Cd may not be as influential to bacterial community shifts than other measured characteristics but could be important to bacterial mat growth as Cd was significantly higher (p= 0.022) at stations with mats (Table 2.8). Biomarker analyses from Verhoeven et al. (2018) indicated that Recently disturbed and High Impact stations exclusively shared only 2/19 biomarker members; *Marinifilum* and *Sphaerochaeta*, excluding genera shared among the three clusters (High Impact, Recently Disturbed and Intermediate Impact) tested in the biomarker analysis. Interestingly, Verhoeven et al. (2016) found a relatively high abundance of both *Spirochaeta* and *Marinifilum* near cage edge where OE is highest at a 3-month fallow site in NL. Compared to High Impact stations, Recently Disturbed stations also contained anaerobic bacteria that have been discovered in deep methane seeps (genera *Izimaplasma;* Skennerton et al., 2016). These large differences between High Impacted and Relatively Disturbed communities could be explained by the variability in measured sediment characteristics.

Less impacted stations (Recently Disturbed and Intermediate stations) indicated an increase in Proteobacteria biomarkers (Verhoeven et al., 2018). In fact, the presence of Proteobacteria increased when marine sediment at a fish farm site in Japan became less enriched with the introduction of a polychaete species (Kunihiro et al., 2008).

According to the Kruskal-Wallis analysis, there were no significant differences among sediment characteristics for Low Impact (including reference stations) and Intermediate Impact stations. This was also reflected in the PCA as this pair grouped closely together and were also the least different among clusters compared to High Impact and Low Impact stations. These results suggest that communities and conditions that dominate fallow sites (Intermediate and Low Impact stations) are more similar than those that dominate production sites (High Impact and Recently Disturbed stations).

3.4.4 Conclusions

Our work has identified linkages between bacterial community cluster identity and abiotic sediment characteristics at NL aquaculture sites. While production, fallow and reference sites had significantly different sediment abiotic characteristics, the latter also differed significantly according to bacterial community clusters. In particular, stations with High and Low Impact communities differed significantly in their concentrations of %TOC, %OM and Zn, which are indicators of aquaculture waste. Microbial communities likely reflect environmental change at aquaculture sites with greater sensitivity than the abiotic characteristics measured here; changes in microbial communities along with distance to cage or during fallow were evident while spatial/temporal patterns in abiotic characteristics were elusive.

We also gained insights into bacterial mat dynamics: mats appeared at the onset of production and appeared to become denser over time during a production cycle. In contrast, at the fallow site, the visual appearance and density of mats remained unchanged, regardless of its bacterial community makeup or duration of fallow. Notably, the visual appearance of mats (i.e. their apparent density) does not relate to microbial community composition; this information is of importance for the proper interpretation of enrichment and recovery status in regulatory frameworks.

Our results suggest that the interpretation of ecosystem status based on the presence of bacterial mats may not be as straightforward as expected, given that mat presence occurred over a broad range of sedimentary conditions and in association with various bacterial communities. Future studies could explore alternative, less subjective approaches for identifying microbial cluster identity based on visual features (for example, using machine learning) and investigate relationships with other visual OE indicators such as OPCs.

3.5 References

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3.6 Appendices

Appendix D: Station details

Table D.1. Details for bacterial swabs taken at each station with assigned impact cluster.

Station #	Site Status	Distance from cage (m)	Transect	Sample Year	Sample ID	Impact	Mat Presence
1	fallow	0	3S	2016	AQ1	Low Impact	Y
2	fallow	0	3S	2016	AQ2	Intermediate Impact	Y
3	fallow	20	3S	2016	AQ3	Low Impact	Y
3	fallow	20	3S	2016	AQ4	Low Impact	Y
3	fallow	20	3S	2016	AQ5	Low Impact	Y
4	fallow	40	3S	2016	AQ6	Intermediate Impact	Y
4	fallow	40	3S	2016	AQ7	Intermediate Impact	Y
4	fallow	40	3S	2016	AQ8	Intermediate Impact	Y
5	fallow	80	3S	2016	AQ10	Low Impact	Y
5	fallow	80	3S	2016	AQ11	Low Impact	Y
5	fallow	80	3S	2016	AQ9	Low Impact	Y
6	fallow	120	3S	2016	AQ12	Low Impact	Y
6	fallow	120	3S	2016	AQ13	Low Impact	Y
6	fallow	120	3S	2016	AQ14	Low Impact	Y
7	fallow	160	3S	2016	AQ15	Low Impact	N
7	fallow	160	3S	2016	AQ16	Low Impact	N
7	fallow	160	3S	2016	AQ17	Low Impact	N
8	fallow	0	8W	2016	AQ19	High Impact	Y
8	fallow	0	8W	2016	AQ20	High Impact	Y
8	fallow	0	8W	2016	AQ21	High Impact	Y
9	fallow	40	8W	2016	AQ22	Low Impact	N
9	fallow	40	8W	2016	AQ23	Low Impact	N

9	fallow	40	8W	2016	AQ24	Low Impact	N
10	fallow	80	8W	2016	AQ25	Low Impact	N
11	fallow	0	4W	2016	No nucleic acid extraction	N/A*	Y
14	production	20	4S	2016	AQ30	Recently Disturbed	Y
14	production	20	4S	2016	AQ31	Recently Disturbed	Y
14	production	20	4S	2016	AQ32	Recently Disturbed	Y
15	production	0	X2	2016	AQ44	High Impact	Y
16	production	5	1N	2016	AQ41	Recently Disturbed	Y
16	production	5	1N	2016	AQ42	Recently Disturbed	Y
16	production	5	1N	2016	AQ43	Recently Disturbed	Y
21	production	0	4W	2016	AQ35	Recently Disturbed	Y
21	production	0	4W	2016	AQ36	Recently Disturbed	Y
21	production	0	4W	2016	AQ37	Recently Disturbed	Y
22	production	0	4S	2016	AQ27	High Impact	Y
22	production	0	4S	2016	AQ28	High Impact	Y
22	production	0	4S	2016	AQ29	High Impact	Y
23	production	20	4W	2016	AQ38	High Impact	N
23	production	20	4W	2016	AQ39	High Impact	N
23	production	20	4W	2016	AQ40	High Impact	N
29	reference	500	Ref3	2017	AQ106	Low Impact	N
29.2	reference	500	Ref3	2017	AQ107	Low Impact	N
30	fallow	0	4W	2017	AQ108	Intermediate Impact	Y
31	fallow	0	3S	2017	AQ50	High Impact	Y
31	fallow	0	3S	2017	AQ51	High Impact	Y
32	fallow	20	3S	2017	AQ52	Intermediate Impact	Y
33	fallow	40	3S	2017	AQ53	Intermediate Impact	Y
33	fallow	40	3S	2017	AQ54	Intermediate Impact	Y
34	fallow	40	3S	2017	AQ55	High Impact	Y

35	fallow	80	3S	2017	AQ57	Intermediate Impact	Y
36	fallow	120	3S	2017	AQ59	High Impact	Y
36	fallow	120	3S	2017	AQ60	High Impact	Y
37	fallow	160	3S	2017	AQ61	Low Impact	N
37	fallow	160	3S	2017	AQ62	Low Impact	N
38	fallow	80	8W	2017	AQ63	Low Impact	N
39	fallow	40	8W	2017	AQ64	Low Impact	N
40	fallow	20	8W	2017	AQ65	Low Impact	Y
40	fallow	20	8W	2017	AQ66	Low Impact	Y
41	fallow	0	8W	2017	AQ67	Intermediate Impact	Y
42	fallow	120	10S	2017	AQ68	Low Impact	N
42	fallow	120	10S	2017	AQ69	Low Impact	N
43	production	0	1N	2017	AQ70	High Impact	Y
44	production	0	1N	2017	AQ71	High Impact	Y
44	production	0	1N	2017	AQ72	High Impact	Y
45	production	20	1N	2017	AQ73	High Impact	Y
45	production	20	1N	2017	AQ74	High Impact	Y
46.1	production	40	1N	2017	AQ75	Intermediate Impact	Y
46.2	production	40	1N	2017	AQ76	Recently Disturbed	Y
47	production	40	4S	2017	AQ77	Recently Disturbed	Y
48	production	0	Center Cage	2017	AQ78	Recently Disturbed	Y
49	production	0	Center Cage	2017	AQ79	Recently Disturbed	Y
53	production	0	4W	2017	AQ80	N/A	Y

N/A= sample was not assigned to a group due to low read count. N/A*= Nucleic acid extraction not performed.

Chapter 4. Summary and conclusions

As Newfoundland (NL) continues to expand its salmonid aquaculture industry throughout the province, it is important to ensure its environmental sustainability. The provincial and federal governments play an important role in this effort as they provide support for furthering research and development to improve environmental monitoring and evaluation of aquaculture sites (NLDFA, 2014; DFO, 2018). Organic enrichment (OE) generated during production cycles has been identified as a major sustainable management issue with action plans in place to mitigate and monitor its interaction with the benthic environment (NLDFA, 2014; DFO, 2018). Monitoring of OE typically consists of measuring sediment parameters (i.e. free sulfide and redox potential) via grab sampling but these methods are limited to naturally soft-bottom sites mostly found in other provinces (Hamoutene et al., 2013). Due to NL's hard-bottom substrates containing very little natural sediment, monitoring efforts alternatively use video surveys to document biotic indicators of OE, notably white bacterial mats, around cages (DFO, 2012, 2015). While this method is useful and informative, there remain important knowledge gaps concerning relationships between bacterial mat presence and geochemical changes within the substrate over space and time, limiting the level of confidence when interpreting local ecosystem status where these mats are present. Previous studies have shown that identifying bacterial assemblages using high-throughput sequencing technology can be beneficial for routine monitoring of OE at aquaculture sites (Verhoeven et al., 2016, 2018; Keeley et al., 2018), but clear relationships between bacterial community composition and the presence of visual

indicators have yet to be established. In NL, recent research regarding OE at aquaculture sites has investigated abiotic sediment characteristics (Anderson et al., 2005; Hamoutene, 2014; Hamoutene et al., 2018) and bacterial communities in sediments and flocculent matter near cages (Verhoeven et al., 2016, 2018), providing the foundation for the present effort to better link bacterial mats and both sediment abiotic and biotic features.

4.1 Relationships between bacterial mats and sediment abiotic characteristics

The primary aim of Chapter 2 was to examine relationships between bacterial mats and abiotic characteristics of the underlying substrate at NL aquaculture sites. Additionally, trends in the measured abiotic features with distance to cage during production and fallow periods, and trends over time during fallowing, were explored. As expected, the analysis of grab samples revealed that hard-bottom dominated aquaculture sites off the south coast of NL were experiencing OE, most likely due to fish feces and fish pellets that collected onto the seafloor during production. Organic enrichment was evidenced by elevated levels of %OM, %TOC and %TN near cages; in agreement with previous studies, the %OM decreased with distance from cages (Ye et al., 1991; Karakassis et al., 2000; Carroll et al., 2003; Sarà et al., 2004; Corner et al., 2006). The organic components were also significantly, positively correlated with many other sediment parameters measured (P, Ca, Zn and Cu), as reported at other aquaculture sites as per husbandry practices (Chou et al., 2004; Sutherland et al., 2007). Despite the low abundance of natural sediments and the likely predominance of aquaculture-derived sedimentary deposits at our stations (especially those closer to cages), the patterns in P, Ca, Zn and Cu concentrations are nevertheless consistent with those observed elsewhere. Our results therefore highlight the potential usefulness of P, Ca, Zn and Cu as markers of aquaculture activity in the NL region. Further

work, examining a greater number of samples from a broader range of conditions, would be required in order to establish useful thresholds for management purposes.

Bacterial mat presence was linked with OE, as was observed previously in NL (Hamoutene et al., 2013, 2016; Hamoutene, 2014; Verhoeven et al., 2016; Salvo et al., 2017). Mats were present at most study stations up to 120 m from cages, in both production and fallow conditions, and regardless of sampling date. Those results agree with other NL studies reporting that mats were most commonly found within 10 m from cages and up to 100 m away from them, and that mats can persist into fallow periods of at least 15 months (Hamoutene et al., 2016; Salvo et al., 2017). We found that mat presence was associated with a broad range of sediment concentrations for most abiotic characters considered, suggesting that mats can become established atop sediments with divergent abiotic characters (i.e. elemental and organic content), and that the composition of mat bacteria may differ across stations. However, stations with and without mats differed significantly in %OM, Zn, Cd and Cu, providing further evidence that mats are linked to aquaculture waste (supporting Hamoutene et al., 2015, Crawford et al., 2001) and providing some indications of the ideal sedimentary conditions for mat formation. Based on our data, there may be a possible OM threshold between 4.3 and 9.8 %OM under which bacterial mats cannot form (or be visible), at least in our region.

Although we had little data from which to explore changes in sedimentary features and bacterial mat presence over a 10 month fallowing period, we did not observe the expected decrease in OM concentrations or in bacterial mat presence, suggesting a slow recovery. Correspondingly, abiotic sediment characteristics remained relatively unchanged over this period. The non-recovery might be linked to low temperature at depth and the persistence of soft substrates.

4.2 Relationships between bacterial communities and both mat presence and abiotic sediment characteristics

Building upon the previous characterization of four bacterial community clusters from sediments retrieved from NL aquaculture sites in Verhoeven et al. (2018), we explored relationships between predefined bacterial assemblages and both bacterial mat presence and sediment abiotic measurements during production and fallow periods (Chapter 3). We considered drop-camera video data along with bacterial community cluster identity and found mats associated with each of the four bacterial community types. As our study was not designed to identify the specific bacteria that form mats at the sediment surface, it is not known whether mat-forming bacteria were of the same species at all stations where they were observed; however, we noted that some bacteria belonging to the taxa Deltaproteobacteria, *Spirochaeta*, Cloacimonadales and Bacteriodales were significantly associated with mat presence.

Our results lead us to conclude that a visible bacterial mat at a NL aquaculture site is not representative of a narrow and highly specific set of biotic traits with regards to the bacterial community. Instead, bacterial mats could signal various stages of aquaculture-linked enrichment within the sediment ("Recently Disturbed", "Low Impact", "Intermediate Impact" or "High Impact"). Therefore, mat presence alone is of restricted utility as a marker of enrichment or remediation stage, as suggested previously in our region (Salvo et al., 2017). Moreover, mats did not visually differ across stations containing distinct bacterial community clusters, but appeared instead to change over time at production and fallow stations, with presumed initial growth stages visible during production and mat stabilization occurring during fallow periods.

We also examined sediment abiotic characteristics alongside bacterial community composition and found while sediment characteristics varied across stations sharing a particular bacterial community cluster, certain patterns emerged while comparing across clusters. Most notably, stations with High Impact communities had significantly greater %OM, %TOC and Zn concentrations than stations with Low Impact communities. Highly impacted communities were dominated by sulfate-reducing anaerobic bacteria from the Phyla Bacterioidetes, Firmicutes and Spirochaetes (Verhoeven et al., 2018), indicating, especially near cages, that sediment conditions were most likely oxygen-deprived. Lesser impacted stations at the fallow site were defined by an increase in Proteobacteria members (Verhoeven et al., 2018) with a relatively low variation in sediment abiotic parameters. Overall, bacterial communities appeared to be more sensitive indicators of environmental change at aquaculture sites than did the abiotic sedimentary features considered in this study.

Interestingly, more than half of the stations sampled at the fallow site shifted towards higher impacted communities over time, illustrating how varied and complex remediation can be, as seen in other studies (Lumb, 1989; Ritz et al., 1989, Karakassis et al., 1999; Wan Hussin et al., 2012; Keeley et al., 2014).

4.3 Conclusions and future directions

In conclusion, sediment OE from salmonid aquaculture can heavily change and influence benthic environments at farms in NL. While there was variability in both sediment abiotic concentrations and bacterial communities at stations that we sampled, general trends showed that OE is prominent during production periods and can persist into fallow periods. We also noted that bacterial communities and bacterial mats, to some

degree, are related to OE and may transition over time as changes to sediment conditions occur. In our study, site recovery appeared to occur at a slow rate and the length of recovery is hard to determine since it is dependent on many factors. Our work highlights the importance of routine monitoring in NL at each phase of aquaculture to document changes in the benthic habitat; future research should expand on this study's temporal sampling plan to provide a more detailed timeline of remediation in this region.

Our work helps refine our understanding of the abiotic sediment conditions present where bacterial mats are observed, thereby strengthening future monitoring efforts. It would be interesting to measure dissolved gases (oxygen, sulfide, methane) in sediment porewaters, as they could potentially be valuable indicators of sediment condition. These gases are likely to be more closely tied to bacterial community composition than the parameters we measured in our study. Developments in instrumentation to measure dissolved gases combined with the use of gliders or ROVs to profile gas concentrations near the seafloor could transform environmental monitoring at aquaculture sites.

Future research should seek to identify visible differences among community clusters to help strengthen routine monitoring efforts. While we were unable to visually differentiate between mats associated with the four bacterial community clusters, there may be subtle differences that could be detected using artificial intelligence techniques. It could be worthwhile to examine whether differences in mat colouration, texture or density could be applied to a machine learning framework to differentiate among organic enrichment states, as characterized by benthic community profiling. If successful, machine learning tools could facilitate the classification of the environmental status of aquaculture sites, providing a shortcut to the more resource-intensive bacterial sequencing and community

determination route that currently seems to provide the most sensitive index of benthic habitat condition.

4.4 References

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