MICROBE-CONTAMINANT LINKAGES IN THE UPPER WATERS OF LAKES

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ABSTRACT

The upper water column (<1 m depth) of freshwater lakes, which includes the surface microlayer (SML; <1 mm depth), is an important microbial habitat as well as an accumulation and dissemination site for chemical and microbial contaminants. This doctoral thesis reports novel insights into how the physical structure and functional capabilities of microbial communities can influence the presence of trace metals and health-relevant bacteria in the upper waters (SML and 0.5 m depth) of freshwater lakes. Two physically and geochemically contrasting lake environments, a remote sheltered boreal lake and a higher energy urban beach on Lake Ontario, were investigated to identify system-dependent physical and biogeochemical factors controlling contaminant-relevant microbial characteristics.

The SML was identified as a major site for generation of contaminant-sequestering suspended flocs from a distinct biofilm-forming microbial community over diurnal timeframes via wind and sunlight exposure, with this process being enhanced at the higher energy beach site. More generally, upper waters including the SML were demonstrated to be inhabited by a diverse group of atypical facultative Fe(III)-reducing bacteria (IRB) that exhibited a SML- and lake-specific capacity for solid Fe(III) reduction directly related to floc and Fe(III) availability. Although IRB were hypothesized to be highly resistant to metals and antibiotics relative to other bacteria due to their ability to dissolve metal-rich Fe(III) minerals, this was not found to be the case. Nevertheless, IRB enriched from the SML demonstrated higher antibiotic resistance compared to those from 0.5 m depth and enriched Fe(III)-

reducing communities from both depths harboured resistance-mobilizing genetic elements and included potentially pathogenic bacteria.

Results of this thesis represent new knowledge concerning how microbial communities regulate the presence of contaminants in the upper waters of lakes. This has important implications for assessing the ecological and human health impacts of contaminants in freshwater systems.

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LIST OF ABBREVIATIONS

DOC ..... Dissolved organic carbon

FeOOH ..... Iron oxyhydroxides

INT ..... Iodonitrotetrazolium chloride

IRB ..... Fe(III)-reducing bacteria

PAR ..... Photosynthetically active radiation

POC ..... Particulate organic carbon

PON ..... Particulate organic nitrogen

SML ..... Surface microlayer

TSS ..... Total suspended solids

PREFACE

This thesis consists of three manuscripts for peer-review publication. Chapter 3 has been published as:

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These manuscripts represent the results of research carried out by the author under the supervision of Dr. Lesley A. Warren. The author was responsible for all field- and laboratory-based data collection, data analysis and interpretation, literature review, and writing and revision.

CHAPTER 1: MICROBIAL COMMUNITIES AND CONTAMINANTS IN THE UPPER WATER COLUMN OF FRESHWATER LAKES

1.1 Introduction

The upper water column (<1 m depth) of freshwater lakes, including the surface microlayer (SML) that occupies the topmost millimeter beneath the air-water interface, is an important site for the accumulation and dissemination of health-relevant trace metals and antibiotic resistant and pathogenic bacteria (Wotton and Preston, 2005). These contaminants are introduced to upper waters via atmospheric deposition of metal-rich anthropogenic emissions, metal- and pathogen-containing wastewater inputs (e.g. combined sewer overflows, surface runoff), and upward transport of metals and bacteria from bed sediments and deeper waters. Upper waters are a likely site of direct human exposure to contaminants via recreational or occupational activities (e.g. swimming and boating) as well as exposure among and dissemination by aquatic organisms that inhabit or visit this region (e.g. waterfowl).

A proper evaluation of chemical and microbial contaminants as determinants of water quality and the hazard they pose to human and ecosystem health requires an understanding of the role played by aquatic microbial communities in controlling the presence, accumulation, behaviour, and dissemination of these substances. Specifically, dense microbial structures including biofilms and suspended flocs can appreciably sorb trace metals and provide microenvironments conducive to (1) microbial transformation of metal sorbents (e.g. reductive dissolution of metal-sorbing Fe(III) phases by Fe(III)-reducing bacteria), (2) bacterial acquisition of mobile antibiotic resistance genes, and (3) pathogen survival.

The upper water column, and particularly the SML, is a high energy region that differs from deeper waters and bed sediments in that it is typically highly oxygenated and strongly impacted by the disruptive actions of solar radiation, wind, and precipitation at the water surface. These characteristics are expected to influence the composition, physical structure, and functional capabilities of upper water microbial communities, and thus their interactions with contaminants. Thus, knowledge concerning contaminant-relevant roles of microbial communities inhabiting the upper water column, including how these roles are influenced by system-dependent characteristics (e.g. energy regime magnitude, anthropogenic impact, nutrient availability), can inform the occurrence and behaviour of chemical and microbial contaminants across freshwater systems, and thus their impact on the health of humans and other organisms.

This introductory chapter aims to provide an overview, based on the peer-reviewed scientific literature, of (1) upper water column microbial communities, specifically the distinct bacterioneuston inhabiting the SML, and (2) how the physical structure of these communities and their capacity for Fe(III) reduction is expected to control the presence of trace metals and antibiotic resistant bacteria in upper waters. This chapter will also detail the research objectives of this thesis, which target generation of new knowledge regarding the as of yet poorly understood regulation of trace metals and health-relevant bacteria by microbial communities inhabiting the upper water column of lakes.

1.2 Surface Microlayer (SML)

Due to its interfacial position, the SML is a gateway for atmosphere-hydrosphere contaminant exchange by mechanisms including atmospheric deposition and the rising and

bursting of bubbles from within the water column (Maki and Hermansson, 1994). These processes, coupled with the stabilization afforded by surface tension and presence of metal-sorbing organic (Södergren, 1993; Münster et al., 1997; Baastrup-Spohr and Staehr, 2009) and Fe oxyhydroxide (Mahowald et al., 2009; Boyd et al., 2010) phases and associated nutrients capable of supporting microbial growth, are associated with the enrichment of trace metals (Armstrong and Elzerman, 1982; Wurl and Obbard, 2004) and antibiotic resistant (Hermansson et al., 1987; Jones et al., 1991; Azevedo et al., 2013) and pathogenic (Dutka and Kwan, 1978; Plusquellec et al., 1991) bacteria in the SML relative to proximate underlying waters (i.e. <0.5 m depth).

In marine environments, SML bacteria (bacterioneuston) can form particle-associated biofilm communities that are phylogenetically distinct from and more metabolically active than those in underlying waters, particularly under calm weather conditions (i.e. low wind speeds and absence of precipitation) and other low energy conditions (e.g. low current velocities) that reduce mixing with underlying waters (Stolle et al., 2010; Santos et al., 2011; Stolle et al., 2011). Dense and highly active SML microbial communities, exposed to intense solar UV radiation and accumulated toxic metals and organics, are expected to be highly antagonistic (Grossart et al., 2004), include antibiotic-producing species (Hakvåg et al., 2008), and be well suited to horizontal gene transfer (Molin and Tolker-Nielsen, 2003), providing a setting conducive to the acquisition and dissemination of microbial contaminants.

1.3 Suspended Aquatic Floc

Counted among the forms of suspended particulate matter found in aquatic systems, flocs specifically refer to aggregates of organic and inorganic particles colonized by an active,

dense, and intrinsic microbial community (Droppo, 2001; Plach et al., 2011; Elliott et al., 2012). While a biofilm refers generally to any cooperative collection of microorganisms anchored to an underlying substratum, flocs are heterogeneous particles, constructed via various physical, chemical, and biological processes, which include a biofilm within their structure. As they are mobile within aquatic systems, flocs provide a vehicle for the transport of contaminants within the water column and to and from bed sediments. Suspended flocs are ubiquitous in aquatic systems and include a biogeochemically potent microbial community (Simon et al., 2002), making them key hotspots for microbe-contaminant interactions in the upper water column. Two fundamental and relatively abundant floc components derived in part from the actions of floc microorganisms, organic matter and Fe oxyhydroxides, are high capacity metal sorbents that enable the scavenging of trace metals by flocs such that their concentrations exceed those found in bed sediments and the surrounding aqueous phase (Plach et al., 2011; Elliott et al., 2012). With respect to microbial contaminants, flocs are well suited to fostering indigenous or introduced bacterial pathogens (Lyons et al., 2010) and can shield them from environmental stresses (e.g. low nutrient availability, solar UV radiation) (Tang et al., 2011). Further, dense microbial communities such as those found in flocs are amenable to the acquisition and dissemination of antibiotic resistance genes (Molin and Tolker-Nielsen, 2003).

The generation of contaminant-modulating flocs is expected to vary throughout the water column as the result of differing availability of floc building blocks and the relative impact of various drivers of aggregation. In particular, wind shear and convective mixing preferentially influence floc generation in upper waters by increasing the frequency with which progenitor particles collide while hindering floc growth under higher energy

conditions (Zimmermann-Timm, 2002). Further, the increased availability of floc ingredients in the form of bacteria, nutrients, and other organic matter in the SML (Crawford et al., 1982; Södergren, 1993; Münster et al., 1997; Baastrup-Spohr and Staehr, 2009) indicates that it is an important site for floc generation within upper waters, but this remains to be investigated.

1.4 Bacterial Fe(III) Reduction

In aquatic systems, Fe(III)-reducing bacteria can reductively dissolve widely available metal-sequestering Fe oxyhydroxides (FeOOH), mobilizing trace metals and thus increasing their bioavailability (Zachara et al., 2001). These bacteria, being O_2-sensitive, have traditionally not been considered to contribute appreciably to Fe transformations in oxic upper waters. This stands in contrast to their well-established role in anoxic deep waters and bed sediments (Lovley, 2013). However, due to the presence of microbially generated O_2-depleted microenvironments within flocs (Paerl and Pinckney, 1996; Simon et al., 2002), Fe(III)-reducing bacteria can operate under bulk oxic conditions (Balzano et al., 2009; Lu et al., 2013; Elliott et al., 2014) such as those found in the upper water column of freshwater lakes. Although not well characterized to date, the oxic and labile organic carbon-rich setting of upper waters, particularly the SML, and distinct nature of suspended floc-based microbial Fe cycling (Elliott et al. 2014) point to a differentiated ecology of Fe(III) reducers in upper waters versus underlying anoxic environments, including with respect to their impact on trace metal mobilization via FeOOH reduction. The SML accumulates organic matter, nutrients, and Fe, the substrates necessary to support microbial Fe(III) reduction, but the

presence and activity of Fe(III)-reducing bacteria, indicative of their influence on accumulated trace metals, in this compartment is not yet known.

In oxic upper waters, floc-based Fe(III)-reducing bacteria are likely highly exposed to trace metals due to their close proximity to, and extremely localized dissolution of trace metal-sequestering FeOOH (Lower et al., 2001; Gorby et al., 2006). Flocs are expected to be particularly amenable to this process due the co-occurrence of Fe reducing and oxidizing bacteria (Elliott et al., 2014) capable of generating highly metal rich amorphous Fe phases and ensuring long term high level metal exposure by cycling Fe between solid and solution phases with concurrent metal uptake and release. Metal stress among floc Fe(III)-reducing bacteria is expected to promote their development of greater metal resistance, as well as greater antibiotic resistance due to the co-selection of these phenotypes within environmental bacterial communities (Baker-Austin et al., 2006). Given their potential antibiotic resistance, Fe(III)-reducing bacteria inhabiting the upper water column of freshwater lakes are hypothesized to represent an important microbial contaminant reservoir in this setting, but this remains poorly understood.

1.5 Research Objectives

The overarching aim of this thesis was to investigate under-characterized contaminant-relevant roles of microbial communities inhabiting the upper water column of freshwater lakes, particularly the SML, and how these roles are influenced by system-dependent physical, geochemical, and microbial factors. Specifically, this work was intended to couple field- and laboratory-based research of two physically and geochemically contrasting lacustrine environments to improve understanding of how microbial

communities in oxic upper waters contribute to floc generation and Fe(III) reduction, and how their involvement in these processes affects their resistance to trace metals and antibiotics. In addition, by evaluating the characteristics of each study site with respect to their impact on the composition, physical structure, and metabolic capabilities of upper water column microbial communities, this thesis aimed to identify system-dependent factors influencing microbially-driven floc generation, Fe(III) reduction, and chemical resistance, thus providing a cross-system view of these roles. The findings of this thesis are expected to highlight the upper water column of freshwater lakes, and specifically the SML, as being host to a largely underappreciated and biogeochemically robust microbial community, with important implications for understanding the behaviour of floc-associated trace metals and health-relevant bacteria within aquatic systems as a whole.

The objectives of this thesis were as follows:

Objective #1 (addressed in Chapter 3):

Examine the role of microbial communities and associated geochemical parameters in the generation of contaminant-sequestering flocs in the upper waters (SML and 0.5 m depth) of freshwater lakes over diurnal timeframes and across two physically and geochemically contrasting littoral environments.

More precisely, this field-based study aimed to (1) investigate the structural and compositional distinctiveness of the SML microbial community as it relates to floc generation, and (2) establish the impact of system-specific energy regime magnitude and

availability of organic carbon, nutrients, and microorganisms on SML floc generation, by characterizing a high energy beach (Sunnyside Beach) on Lake Ontario and a small sheltered Canadian Shield lake (Coldspring Lake).

Objective #2 (addressed in Chapter 4):

Characterize Fe(III)-reducing bacteria in the upper waters (SML and 0.5 m depth) of two physically and geochemically contrasting littoral freshwater environments with respect to their composition and capacity for reductively dissolving trace metal-sorbing Fe(III) oxyhydroxides, and identify system-specific factors including floc availability that influence these characteristics.

More precisely, this combined field- and laboratory-based study aimed to (1) investigate whether Fe(III)-reducing bacteria occur in the SML and proximate underlying oxic waters (0.5 m depth) of freshwater lakes (Coldspring Lake and Sunnyside Beach on Lake Ontario), (2) identify, if present, differences in the composition of Fe(III)-reducing bacterial enrichments and their capacity for soluble and solid phase Fe(III) reduction between depths and systems, and (3) assess how lake-specific level of anthropogenic impact, degree of bed sediment resuspension, and availability of organic carbon, nutrients, and Fe influence the composition and capacity of Fe(III)-reducing enrichment communities.

Objective #3 (addressed in Chapter 5):

Evaluate the relative trace metal and antibiotic resistance, and associated health-relevant characteristics, of Fe(III)-reducing members of bacterial communities enriched from the

upper waters (SML and 0.5 m depth) of two physically and geochemically contrasting littoral freshwater environments.

More precisely, this study aimed to (1) assess the trace metal and antibiotic resistance of Fe(III)-reducing bacteria relative to non-Fe(III)-reducing members of soluble Fe(III)-reducing communities enriched from the SML and proximate underlying waters (0.5 m depth) of a contaminated urban beach (Sunnyside Beach) and a pristine remote lake (Coldspring Lake), and (2) investigate the relative health relevance of soluble Fe(III)-reducing communities between depths and systems based on their level of antibiotic and trace metal resistance and inclusion of mobile genetic elements capable of disseminating resistance genes.

CHAPTER 2: FIELD SITES, WATER SAMPLING AND ANALYSIS, MICROBIAL ENRICHMENT, AND LABORATORY EXPERIMENTS

2.1 Introduction

For this thesis, field- and laboratory-based methods were used to investigate how upper water column microbial communities carry out contaminant-relevant floc generation and Fe(III) reduction, and identify how these microbial processes are influenced by system- and depth-dependent physical, chemical, and microbial factors. This chapter is intended to provide (1) greater detail concerning the two investigated field sites than was stated in the three results chapters (Chapter 3-5), and (2) a synthetic overview and justification of the methods employed to collect and analyze water samples and characterize enriched Fe(III)-reducing communities. Additional details regarding the sampling and analysis of water samples and experimental characterization of Fe(III)-reducing communities can be found in the results chapters.

2.2 Field Sites

Field sampling (*in situ* water column characterization and water sample collection) was carried out at two contrasting freshwater littoral sites in different temperate freshwater lakes in Ontario, Canada over diurnal timeframes in consecutive summers (2010 and 2011). The two sites differed with respect to their physical structure, energy regime magnitude, water chemistry, and degree of anthropogenic impact (Figure 2.1).

Coldspring Lake is a relatively small (<1 km^2) and shallow (mean depth ~3 m, maximum depth ~7 m) wetland-associated highly organic and Fe-rich, soft water Canadian

Shield lake sheltered by bordering dense forest. The lake is located in a remote northwestern region (45°51'14"N 78°49'40"W) of Algonquin Provincial Park accessible only by floatplane and designated as a nature reserve zone where campsite development is prohibited (Ontario Ministry of Natural Resources, 1998). As such, it is considered to be relatively pristine.

Sunnyside Beach is a 1.7 km long relatively organic- and Fe-poor manufactured sand beach on the northwestern shore of Lake Ontario (~19 000 km^2) (43°38'14"N 79°27'18"W) in the heavily urbanized west part of downtown Toronto (population ~3 million), impacted by bird fecal contamination, municipal wastewater (untreated and treated effluent), urban runoff, and traffic- and industry-sourced air pollution (Edge et al., 2010; Nazzal et al., 2013). Specifically, the beach is located several hundred meters from a major highway (the Gardiner Expressway) and <5 km from a major wastewater treatment plant (Humber Treatment Plant) and the outflows of the Humber River and Mimico Creek, which drain urban and agricultural lands and receive combined sewer overflows that occasionally include untreated wastewater (City of Toronto, 2014a). During the summers of 2010 and 2011 when this site was sampled, *Escherichia coli* concentrations occasionally exceeded 100 cells per 100 mL of water at the beach, necessitating its closure in accordance with the provincial standard (City of Toronto, 2014b). Otherwise, the beach is available to the public and is used recreationally for swimming and boating.

Although sampling sites at both systems were within the shallow littoral zone, the water column depth was ~3 m at Coldspring Lake and ~1 m at Sunnyside Beach. Further, the fetch at Sunnyside Beach was >50 km based on the south/west wind direction at time of sampling, compared to <1 km at Coldspring Lake. The two contrasting field sites were chosen to best assess the system dependence of contaminant-influencing microbial

community characteristics and thereby identify specific physical, geochemical, and microbial factors that influence these characteristics. Specifically, the two sites permitted evaluation of the influence of energy regime magnitude (i.e. fetch and degree of wind and sunlight exposure) and availability of floc ingredients (organic carbon, microbes, nutrients, and Fe) on floc generation (Chapter 3), the availability of floc, Fe, nutrients, and organic carbon on microbial Fe(III) reduction (Chapter 4), and degree of anthropogenic impact on the level of trace metal and antibiotic resistance in Fe(III)-reducing enrichment communities (Chapter 5).

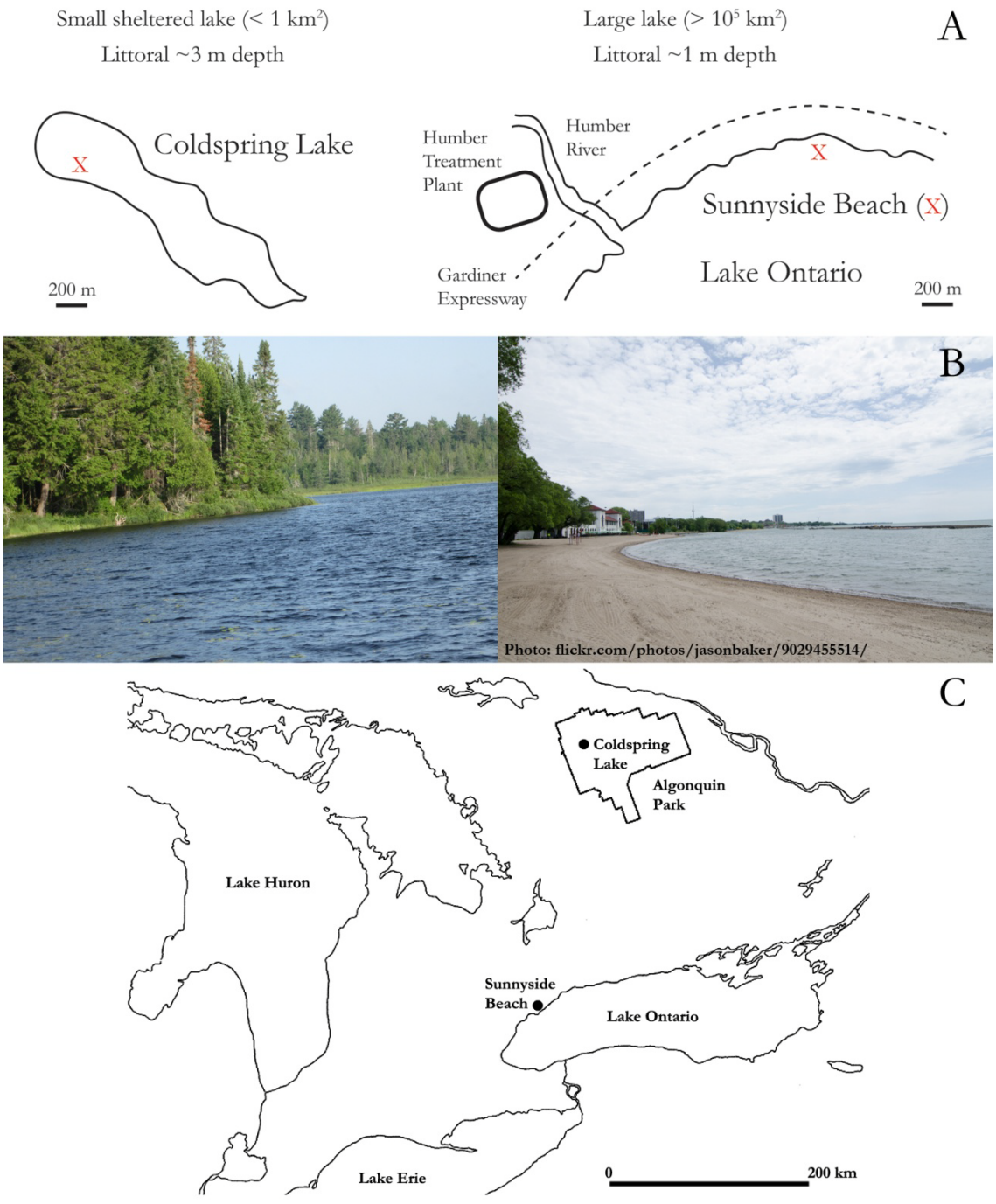

Figure 2.1 Field sampling (*in situ* water column characterization and water sample collection) was carried out at two contrasting freshwater littoral sites (Coldspring Lake and Sunnyside Beach on Lake Ontario, locations indicated by red Xs) over diurnal timeframes in consecutive summers (2010 and 2011). (A) Local site maps, (B) site photographs, and (C) regional scale map.

2.3 Site Characterization and Water Sample Collection

Water sample collection and *in situ* characterization of water column physico-chemistry and site meteorological conditions was undertaken throughout the day in the summers of 2010 and 2011 (Table 2.1). At Sunnyside Beach, diurnal sampling was carried out over a single day, while at Coldspring Lake it occurred over a two day excursion (afternoon then morning of the next day) due to logistical constraints. This approach permitted the capture of diurnally variable meteorological conditions and water column physico-chemical and biogeochemical conditions expected to influence floc generation and the composition and function of Fe(III)-reducing bacteria, while also providing a larger number of discrete water samples for the enrichment, and subsequent characterization, of Fe(III)-reducing communities. Sample collection and *in situ* physico-chemical characterization was carried out according to established field protocols. At each site, physico-chemical characteristics (°C, pH, dissolved O_2 concentration) of the SML and at 0.5 m water column depth were directly characterized. Underwater photosynthetically active radiation (PAR, 400-700 nm) was measured to establish the littoral nature of the sample collection locations (i.e. >1% of PAR irradiance reached the sediment-water interface at both sites). Solar UV irradiance and wind speed data were obtained to establish the energy regime magnitude for each site during sampling periods. In 2011, diurnal variation in solar UV irradiance including its peak magnitude was measured at the air-water interface of both sites. Wind speed and direction (hourly observations) during sampling periods in both years were obtained from the National Climate Data and Information Archive (Environment Canada, 2011) for the nearest monitoring station.

Table 2.1 Dates and times of *in situ* water column characterization and water sample collection.

Field site	Year/Month/Day	Time	Samples collected
Coldspring Lake	2010/08/11	15:00	*In situ* characterization
			Bulk water [a]
	2010/08/12	07:00	*In situ* characterization
			Bulk water [a]
			Slide samplers
			Filters for DNA and enrichment
		13:00	*In situ* characterization
			Bulk water [a]
			Slide samplers
			Filters for enrichment
	2011/07/20	12:00	Bulk water
		16:00	Bulk water
		19:00	*In situ* characterization
			Bulk water
			Slide samplers
			Filters for enrichment
	2011/07/21	06:00	*In situ* characterization
			Bulk water
			Slide samplers
			Filters for enrichment
Sunnyside Beach	2010/07/21	10:00	*In situ* characterization
			Bulk water [a,b]
			Slide samplers
			Filters for DNA and enrichment
		15:00	*In situ* characterization
			Bulk water [a,b]
			Slide samplers
			Filters for enrichment
	2011/08/10	10:00	*In situ* characterization
			Bulk water
			Slide samplers
			Filters for enrichment
		13:00	Bulk water
		15:00	*In situ* characterization
			Bulk water
			Slide samplers
		18:00	Bulk water
		20:00	*In situ* characterization
			Bulk water

[a] Included containers for analysis of microbial abundance and floc size and abundance

[b] Included containers for analysis of organic carbon and organic nitrogen at the National Laboratory for Environmental Testing

Water samples were collected at both sites from the littoral region of the lake, ~5 m from the shore at Sunnyside Beach and ~15 m from the shore at Coldspring Lake. Samples were collected using four different sampling tools (sheet sampler, Van Dorn bottle sampler, slide samplers, and membrane filters) for analysis of trace metals, nutrients, organic carbon and nitrogen, suspended floc size and abundance (all using bulk water samples), and microbial community physical structure (DAPI-based fluorescent nucleic acid imaging of slide samplers), composition (16S rRNA gene analysis and enrichment of Fe(III)-reducing communities from membrane filters), and abundance (cell counting of bulk water samples) (Figure 2.2).

To minimize organic, nutrient, and trace metal contamination, all equipment (excluding slide samplers and membrane filters, which were maintained under sterile conditions until use) and containers used for the collection and storage of water samples were cleaned via a three step process before use: (1) washing with Extran 300 detergent and rinsing with ultrapure water (18.2 MΩ cm), (2) soaking in a 4% (v/v) HCl solution prepared using ultrapure water for >24 h (exception: 10% (v/v) H_2SO_4 for bottles used for measuring DOC at the National Laboratory for Environmental Testing), and (3) rinsing eight times with ultrapure water. For larger equipment (i.e. sheet sampler and Van Dorn bottle sampler), only surfaces in direct contact with the collected water sample were thoroughly cleaned. In the field, containers were rinsed at least twice with lake water before being filled and closed. Operational field blanks of ultrapure water were also assessed and indicated negligible contamination for each geochemical and microbial analyte.

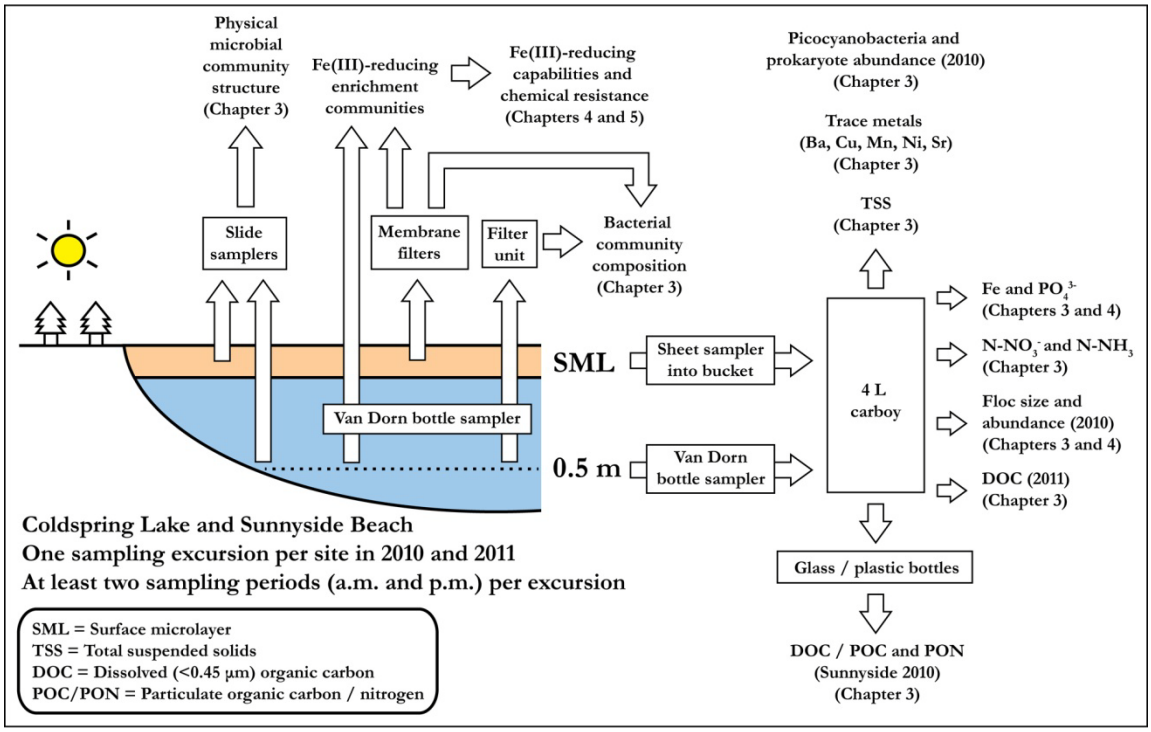

Figure 2.2 Sampling devices employed to collect water from the SML and at 0.5 m depth at each field site for the analysis of geochemical and microbial parameters.

To collect a sufficient volume of water (~2.5-3 L) from the SML for the analysis of a suite of biogeochemical parameters in a minimal amount of time, a sheet surface sampler with a large surface area was designed especially for this thesis and employed to maximize the volume collected per use. Concurrent with SML sampling with the sheet sampler, a water sample (~2.5-3 L) was collected from 0.5 m depth using a clean Van Dorn bottle sampler. Slide samplers, consisting of glass microscope slides coated with a collodion polymer membrane to which biological materials adhere upon contact, were prepared and used according to Henk (2004) to collect samples from the SML and 0.5 m depth for qualitative microscopic examination of microbial community structure. Sampling of the SML microbial

community for analysis of bacterial community composition and the enrichment of Fe(III)-reducing microbial communities was carried out according to Cunliffe et al. (2011) using nucleation-track polycarbonate membrane filters (47 mm diameter, 0.1 μm pore size). To sample the microbial community at 0.5 m depth, water samples were collected using a Van Dorn sampler and filtered through cellulose acetate filters (0.2 μm pore size) that were then excised and placed in sterile Petri dishes.

All samples were stored in coolers with ice packs until such time that they could be placed in refrigerators or freezers for longer term storage (Table 2.2). Samples for trace metal analysis were acidified prior to storage using trace metal grade HNO_3 to minimize any changes from the time of their collection (e.g. microbial growth, trace metal and nutrient sorption/desorption, and organic carbon aggregation). Similarly, samples for measuring microbial abundance were fixed with glutaraldehyde to minimize microbial growth.

2.4 Sample Analysis

Water samples collected from upper waters (SML and 0.5 m depth) were analyzed for geochemical and microbial parameters including the size and abundance of flocs and the presence of Fe(III)-reducing bacteria (Table 2.2). Water geochemistry, floc size and abundance, and the composition, abundance, and physical structure of microbial communities were analyzed to assess their influence on floc generation and characteristics of Fe(III)-reducing bacteria in upper waters (SML and 0.5 m depth) and thus identify factors influencing contaminant-relevant microbial processes in this setting. Concentrations of total Fe (Fe(II) + Fe(III)), nutrients (PO_4^{3-}, NO_3−N, and NH_3−N), dissolved organic carbon (<0.45 μm), particulate organic carbon and nitrogen (>0.45 μm), total suspended solids, and

prokaryotic cells (total and picocyanobacteria) were analyzed because they were known floc components and thus expected to control the extent of floc generation (i.e. floc size and abundance) in upper waters. In particular, photoautotrophic picocyanobacteria are capable of contributing organic matter to floc development, and nutrients and organic matter can support the establishment and development of the floc microbial community. Total suspended solids served as a proxy for bed sediment resuspension and its potential contribution to floc generation. Characterization of the composition and physical structure of microbial communities at each depth permitted an assessment of their propensity toward forming biofilms (i.e. presence of biofilms and known biofilm-associated taxa), from which flocs could be derived. Bacterial 16S rRNA genes were selectively amplified from total community DNA by PCR using a primer set that ensured the capture of all nine variable regions of the gene. Concentrations of trace metals, both conservative (Ba and Sr) and nonconservative (Cu and Ni) with respect to their relative affinity for organics and Fe, were analyzed in order to assess the depth- and site-specific impact of microbial communities including Fe- and organic-rich flocs on the behaviour of trace metals expected to appreciably interact with them in aquatic environments.

Total Fe, nutrients, and organic carbon were also analyzed due to their expected influence on the presence and capabilities of Fe(III)-reducing bacteria, being substrates that they utilize. Floc size and abundance were measured to establish their influence on the presence and capacity of Fe(III)-reducing bacteria, which due to their sensitivity to O_2 were expected to inhabit O_2-depleted internal microenvironments within flocs in otherwise well-oxygenated upper waters. The presence of soluble Fe(III)-reducing bacteria was assessed using an enrichment medium that provided a visual indicator of their growth.

Table 2.2 Water sample processing, storage, and method of analysis for geochemical and microbial parameters expected to influence contaminant-relevant microbial processes in the upper water column of freshwater lakes.

Parameter	Sample processing	Sample storage	Method of analysis
Soluble Fe (Fe(II) + Fe(III)) (dissolved (<0.2 μm) and total) concentration	Sheet or Van Dorn sampler → Carboy (4 L, LDPE) → Syringe with sequential 0.45- and 0.2-μm filters (dissolved samples only) → Falcon tubes (15 mL, PP) → Acidify with trace metal grade HNO_3 (2% (v/v) final)	In the dark at 4°C	Spectrophotometric FerroVer method (1,10-phenanthroline complexation) (Hach Company, Loveland, CO)
Trace metal (Ba, Cu, Mn, Ni, and Sr) (dissolved (<0.2 μm) and total) concentration			Inductively coupled plasma mass spectrometry (Sciex Elan 6100; PerkinElmer, Woodbridge, ON)
Total soluble reactive phosphate (PO_4^{3-}) concentration	Sheet or Van Dorn sampler → Carboy (4 L, LDPE)	In the dark at 4°C	Spectrophotometric PhosVer 3 ascorbic acid method (Hach Company)
Total soluble nitrate (NO_3−N) concentration			Spectrophotometric cadmium reduction method (Hach Company)
Total soluble ammonia (NH_3−N) concentration			Spectrophotometric Nessler method (Hach Company)
Dissolved organic carbon (<0.45 μm) concentration (2010 only)	Sheet or Van Dorn sampler → Carboy (4 L, LDPE) → Glass bottles (120 mL, cleaned with 10% H_2SO_4)	In the dark at 4°C	UV/persulfate oxidation method (Environment Canada, 1979)
Dissolved organic carbon (<0.45 μm) concentration (2011 only)	Sheet or Van Dorn sampler → Carboy (4 L, LDPE)	In the dark at 4°C	Spectrophotometric method based on absorption at 330 nm, adjusted for Fe interference (Moore, 1985)
Particulate organic carbon and nitrogen (>0.45 μm) concentration (2010 only)	Sheet or Van Dorn sampler → Carboy (4 L, LDPE) → Container (500 mL, Teflon)	In the dark at -20°C	Filter known volume onto precombusted glass fiber filter → Rinse collected particulates with 0.3% H_2SO_4 (v/v) to remove inorganics → Measure CO_2 and N_2 released by complete oxidation (Environment Canada, 1979)
Total suspended solids concentration	Sheet or Van Dorn sampler → Carboy (4 L, LDPE)	In the dark at 4°C	Spectrophotometric method based on the absorbance of a homogenized sample at 810 nm (Hach Company)
Floc size and abundance	Sheet or Van Dorn sampler → Carboy (4 L, LDPE) → Falcon tubes (50 mL, PP)	In the dark at -20°C	Filter known volume onto white polycarbonate filter (0.1 μm pore size) → Light microscopy (Leica DMRA microscope; Leica Microsystems Canada, Richmond Hill, ON)) → Image capture using OpenLab software (v 5.1; PerkinElmer, Woodbridge, ON) → Image analysis using ImageJ software (v 1.43) (Abramoff et al., 2004)

Table 2.2 (Continued)

Parameter	Sample processing	Sample storage	Method of analysis
Total prokaryote and phycoerythrin-containing picocyanobacteria cell abundance	Sheet or Van Dorn sampler → Carboy (4 L, LDPE) → Falcon tubes (50 mL, PP) → Fix with glutaraldehyde (1% (v/v) final)	In the dark at -20°C	Filter known volume onto black polycarbonate filter (0.1 μm pore size) → Stain with DAPI (fluorescent nucleic acid stain) → Epifluorescence microscopy (Leica DMRA microscope) → Count stained cells (total prokaryotes) and autofluorescing cells (470 nm excitation, 525 nm emission) (phycoerythrin-containing picocyanobacteria)
Biofilm images (microbial community physical structure)	Slide samplers (Henk, 2004) → Falcon tube (50 mL, PP)	In the dark at 4°C	Epifluorescence microscopy (Leica DMRA microscope) using DAPI
Environmental bacterial community composition	**SML:** Membrane filters → Screw top tube (2 mL, PP) **0.5 m depth:** Van Dorn sampler → Filter unit (0.2 μm pore size, cellulose acetate) → Excise filter and store in sterile Petri dish	In the dark at -80°C	Total community DNA isolation (PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA)) → PCR amplification of bacterial 16S rRNA genes using 27F/1492R primer set → Cloning and isolation of genes (TOPO TA Cloning Kit for Sequencing; Invitrogen, Carlsbad, CA) → Sequencing (MOBIX Lab, McMaster University, Hamilton, ON)
Presence of Fe(III)-reducing bacteria	**SML:** Membrane filters → Falcon tube (15 mL, PP) containing 13 mL of soluble Fe(III) citrate-based enrichment medium **0.5 m depth:** Van Dorn sampler → Falcon tube (15 mL, PP) containing 13 mL of aforementioned medium	In the dark at ~23°C	Visualization of Fe(III)-reducing bacteria growth by clearing of orange-tinted medium (reduction of soluble Fe(III)) and precipitate formation

2.5 Laboratory Experiments Using Fe(III)-Reducing Enrichment Communities

Soluble Fe(III)-reducing communities enriched from upper waters (SML and 0.5 m depth) were subjected to experiments designed to assess their association with trace metal and microbial contaminants by characterizing their bacterial community composition, ability to reduce solid Fe oxyhydroxides (FeOOH), resistance to trace metals and antibiotics, and inclusion of mobile genetic elements (Figure 2.3). To establish the presence of soluble Fe(III)-reducing bacteria in upper waters, SML and 0.5 m depth water samples were used to

inoculate a soluble Fe(III) citrate-based medium (Kostka and Nealson 1998). These bacteria were specifically targeted for enrichment because their clearing of orange-brown soluble Fe(III) citrate from solution and subsequent formation of a brown precipitate provides a clear and relatively rapid visual indicator of their activity. This enabled the detection of their presence in water samples and subsequently the selective assessment of their trace metal and antibiotic resistance in mixed Fe(III)-reducing enrichment communities. To assess the ability of soluble Fe(III)-reducing enrichment communities to also reduce trace metal-sequestering FeOOH solids, as well as their bacterial community composition, experimental microcosms were set up using a growth medium based on a synthetic mixture of amorphous and crystalline FeOOH. Microcosms were inoculated with a previously established soluble Fe(III) citrate-reducing enrichment community and incubated under microoxic (<15% O_2 saturation) conditions. Reduction of FeOOH was quantitatively assessed by the detection of aqueous Fe(II) using the ferrozine method described by Viollier et al. (2000). Concurrent with the measurement of Fe(II) production, bulk enrichment community growth was estimated using a nonselective growth medium that included iodonitrotetrazolium chloride (INT) as a colorimetric indicator of bacterial cell abundance (Hatzinger et al. 2003). To confirm the growth and viability of the microcosm-based enrichment communities and characterize their physical structure, intact (viable) and membrane-compromised microbial cells from microcosms were imaged using the LIVE/DEAD BacLight nucleic acid staining technique. The composition of microcosm bacterial communities was then determined by 16S rRNA gene sequencing.

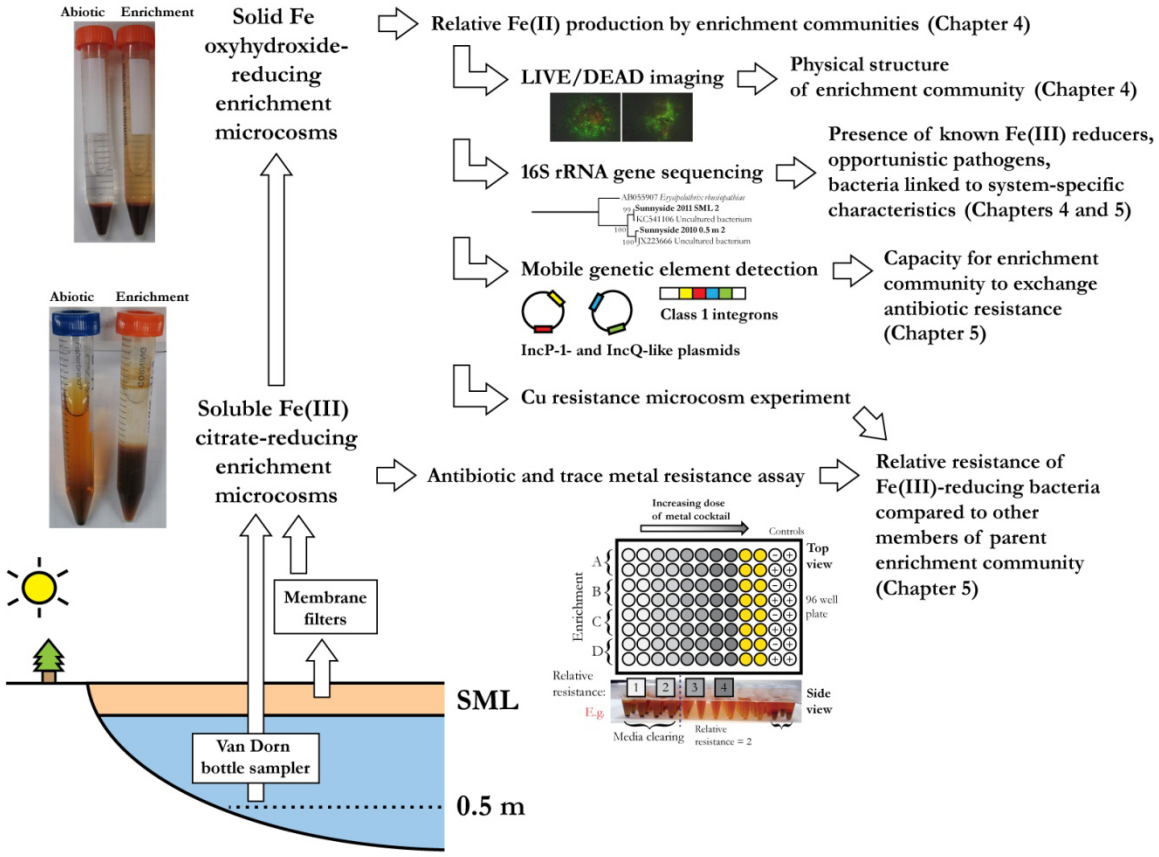

Figure 2.3 Experiments carried out to characterize the composition, physical structure, and functional capabilities of Fe(III)-reducing communities enriched from the SML and at 0.5 m depth.

To assess the trace metal and antibiotic resistance of soluble Fe(III)-reducing bacteria versus other members of soluble Fe(III)-reducing enrichment communities, a 96-well assay was used to determine the inhibition of the metabolic activity of these two groups over a range of concentrations of trace metal or antibiotic cocktails. Soluble Fe(III) reducer activity was visualized using the aforementioned soluble Fe(III) citrate-based enrichment medium. Bulk enrichment community (i.e. soluble Fe(III)-reducing bacteria and other non-Fe(III)-reducing community members) activity was visualized using the aforementioned nonselective medium that included INT, which is reduced to a coloured (violet) product by general

microbial growth. Resistance to trace metals and antibiotics was evaluated using cocktails rather than individual compounds to better represent the exposure conditions for bacterial communities in natural aquatic environments. To assess trace metal resistance, soluble Fe(III)-reducing enrichment communities were exposed to increasing concentrations of a metal cocktail consisting of Ag^{+}, Cd^{2+}, CrO_4^{2-}, Pb^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}. To assess antibiotic resistance, soluble Fe(III)-reducing enrichment communities were exposed to a cocktail of cephalexin, tetracycline, ciprofloxacin, erythromycin, and sulfamethoxazole. Relative resistance, an ordinal scale representing the trace metal or antibiotic cocktail concentration required to inhibit positive visual indicators of the activity of Fe(III)-reducing bacteria or bulk enrichment community activity (lower number = activity inhibited at a lower concentration, i.e. less resistant), was developed for this thesis.

To confirm and expand on the results of the 96-well plate assay, a microcosm-based assay was carried out to determine the relative Cu resistance of solid FeOOH-reducing bacteria compared to soluble Fe(III)-reducing enrichment community members that do not reduce solid Fe(III). This permitted the specific examination of Fe(III) reducers known to be capable of reducing solid Fe(III) and thus expected to be highly resistant to trace metals. Cu was selected as a representative to determine trace metal resistance because it is a common aquatic contaminant known to accumulate in the SML (Armstrong and Elzerman, 1982; Wurl and Obbard, 2004). Further, its high affinity for both organic matter, including microbial cells and their extracellular products, and FeOOH (Dong et al., 2003; Boujelben et al. 2009) makes Cu a highly environmentally relevant candidate for examining the relative metal resistance of solid Fe(III)-reducing bacteria. Microcosms were set up using a growth medium based on the aforementioned synthetic FeOOH mixture. To determine the relative

Cu resistance of solid Fe(III)-reducing bacteria compared to other community members, the activity of solid Fe(III)-reducing bacteria and bulk enrichment community activity were measured in microcosms inoculated with a previously established soluble Fe(III)-reducing enrichment community. Half of the microcosms were treated with Cu prior to inoculation. The reduction of FeOOH, as an indicator of the activity of solid Fe(III)-reducing bacteria, was quantitatively assessed by the detection of Fe(II) at concentrations exceeding abiotic controls during the incubation period. At the end of the incubation period, the bacterial community composition of microcosms treated with Cu was determined by 16S rRNA gene sequencing. The composition of untreated microcosms was determined as part of the aforementioned assessment of the ability of soluble Fe(III)-reducing enrichment communities to reduce FeOOH.

To assess the presence of mobile genetic elements in Fe(III)-reducing enrichment communities, gene cassettes associated with class 1 integrons were selectively amplified from soluble Fe(III)-reducing enrichment community DNA by PCR (Stokes et al., 2001). DNA sequences specific to particular plasmid types (*trfA2* for IncP-1 and *oriV* for IncQ) were also detected in enrichment community DNA by their selective PCR amplification according to Götz et al. (1996).

CHAPTER 3: DIURNAL FLOC GENERATION FROM NEUSTON BIOFILMS IN TWO CONTRASTING FRESHWATER LAKES

3.1 Abstract

Selective adaptation of biofilm-forming bacteria to the nutrient-rich but environmentally challenging conditions of the surface microlayer (SML) or neuston layer was evident in littoral regions of two physically and geochemically contrasting freshwater lakes. SML bacterial communities (bacterioneuston) in these systems were depleted in *Actinobacteria*, enriched in either *Betaproteobacteria* or *Gammaproteobacteria*, and either unicellular *Cyanobacteria* were absent or microbial mat forming *Cyanobacteria* enriched relative to communities in the underlying shallow water column (0.5 m depth). Consistent with the occurrence of biofilm-hosted, geochemically distinct microhabitats, As-, Fe-, and S-metabolizing bacteria including anaerobic taxa were detected only in the SML in both systems. Over diurnal time scales, higher wind speeds resulted in the generation of floc from SML biofilms, identifying a transport mechanism entraining SML accumulated microorganisms, nutrients, and contaminants into the underlying water column. The energy regime experienced by the SML was more important to floc generation as larger flocs were more abundant in the larger, oligotrophic lake (higher relative energy regime) compared to the sheltered, smaller lake, despite relatively higher concentrations of bacteria, organic carbon, Fe, and PO_4^{3-} in the latter system.

3.2 Introduction

Physical and chemical interfaces in freshwater lakes concentrate microbial communities, focusing accumulation and cycling of organic carbon, nutrients, and other metabolic products in these microenvironments.[1−4] In particular, interfaces are important accumulation sites for flocs,[5−7] aggregates of inorganic and organic particles bound together by exopolymer secretions of an intrinsic microbial community,[8,9] which can selectively and significantly impact biogeochemical cycling driven by the high productivity of floc microbial communities,[5] the accumulation of nutrients and contaminants via their sorption to organic and Fe floc components,[9−12] and the transport of accumulated substances within the water column as well as to and from bed sediments.[13,14] Floc generation is expected to vary throughout the water column as the result of differing availability of floc building blocks and the relative impact of known physical, chemical, and biological drivers of aggregation; however, this variation remains poorly characterized.

Located within the initial millimeter of the water column at the air−water interface, the surface microlayer (SML) has been shown to possess distinct physical, geochemical, and biological characteristics compared to adjacent underlying water depths in aquatic systems[15−19] that suggest it may be an important zone for floc generation. Growth of SML bacteria (bacterioneuston) is favored by the accumulation of inorganic and organic nutrients coupled with the stability afforded by surface tension.[15,20−24] Consistent with these factors, higher bacterial abundances and metabolic activities have been observed in the SML relative to underlying waters across a wide range of aquatic systems.[21−27] Consequently, floc generation may be greater in the SML as the result of the increased availability of dissolved organic matter, which can undergo spontaneous aggregation, and bacteria, which can

increase the stickiness of particle surfaces and thus the likelihood of particle aggregation following collision, as floc building materials.[5,28,29] Cunliffe and Murrell[30] proposed the widespread presence of SML aggregates derived from microlayer-enriched organic matter and colonized by biofilm-adapted bacteria. However, the potential advantages of the SML as a microbial habitat are tempered by challenges of exposure to high levels of solar radiation (visible and ultraviolet), accumulated toxic contaminants, and intense wind- and sunlight-sourced hydrodynamic forces.[15−17,31,32] These potentially extreme characteristics suggest the SML contains a distinct microbial community adapted to the particular combination of beneficial and harmful conditions present in the microlayer that distinguish it from the bulk water column, even immediately below it.

Although the composition and functional capabilities of SML microorganisms remain poorly understood, particularly in freshwater systems, cultivation independent investigations have demonstrated that the phylogenetic and functional diversity of SML bacterial communities can differ from subsurface waters in a variety of aquatic environments.[33−39] Additionally, higher metal and antibiotic resistance has been observed for bacteria cultured from the SML compared to underlying waters in both marine and freshwater environments, pointing to important functional differences among microlayer microorganisms.[40−42] These findings suggest that the SML is capable of exerting a characteristic influence on local biogeochemical cycling in the upper water columns of freshwater systems.

Counted among the extreme factors encountered by SML microorganisms, physical forces have an important role in shaping the structure of microbial communities. Turbulent conditions, elevated in the SML due to wind shear force acting at the air−water interface and

convective currents arising from steep temperature gradients that form across the microlayer,[16,17] can promote floc generation by increasing the frequency of collisions between smaller progenitor particles (e.g., scavenging of organic particles by bubbles forming and collapsing at the air−water interface) as well as the efficiency of planktonic exopolymer-producing bacteria attachment to particle surfaces.[5,29,43−46] However, high energy turbulence can also break apart flocs and disrupt their development.[5,47,48] Increased wind exposure has been linked to the disruption of marine particle-based SML bacterial communities,[35,39] implicating wind-driven mixing as a key control on microlayer floc size and abundance.

Physical and biogeochemical factors known to influence floc generation vary among freshwater lakes. System-specific characteristics such as surface area and fetch establish the magnitude of microlayer wind exposure, while morphometry and mean depth affect its degree of interaction with bed sediments in littoral regions, and biogeochemical factors such as concentrations of dissolved organic carbon and specifically bacteria that influence floc generation can differ appreciably among systems. However, studies of the relative influence of physical and biogeochemical factors on SML microbial community characteristics as well as the potential for floc generation, particularly in the context of their variability across freshwater systems, remain scarce.

Thus, the objectives of this field study were to investigate the structural and compositional distinctiveness of the SML microbial community and establish the impact of system-specific physical and biogeochemical factors on SML floc generation, by comparing the SML to the underlying water column (0.5 m depth) in the littoral zones of two highly contrasting freshwater systems, an urban beach on Lake Ontario and a small sheltered lake in a nature reserve.

3.3 Materials and Methods

3.3.1 Description of Field Sites

The two systems chosen for this study differ in a number of physical and biogeochemical characteristics that should influence SML composition and structuring. Sunnyside Beach is a manufactured public sand beach on the northwestern shore of Lake Ontario (~19,000 km^2, 43°38'14"N 79°27'21"W) in downtown Toronto (population ~3 million), impacted by wastewater effluents and traffic- and industry-produced air pollution. Coldspring Lake is a small (<1 km^2), shallow (average depth ~3 m, maximum depth ~7 m) wetland-associated highly organic- and Fe-rich, soft water Canadian Shield lake sheltered by bordering dense forest, in a remote northwestern region (45°51'12"N 78°49'24"W) of Algonquin Provincial Park (Ontario) designated as a nature reserve zone. The fetch at Sunnyside Beach was >50 km based on the south/west wind direction at time of sampling, compared to <1 km at Coldspring Lake. Physicochemical characteristics of each field site are summarized in Table S.3.1.

3.3.2 Sample Collection

Samples were collected at both sites from the littoral region of the lake, ~5 m from the shore at Sunnyside Beach and ~15 m from the shore at Coldspring Lake. At each site, water was sampled from the SML and the underlying water column (0.5 m depth) throughout the day over a one or two day excursion in consecutive summers (2010 and 2011) for analysis of trace elements, nutrients, organic carbon and nitrogen concentrations, suspended floc size and abundance, and microbial community physical structure, composition, and abundance (Figure S.3.1). Coldspring Lake was sampled on August 11 at

15:00 and August 12 at 7:00 and 13:00 in 2010 as well as on July 20 at 12:00, 16:00, and 19:00 and July 21 at 6:00 in 2011. Sunnyside Beach was sampled on July 21, 2010 at 10:00 and 15:00 and on August 10, 2011 at 10:00, 13:00, 15:00, 18:00, and 20:00. Water column physicochemical data (°C, pH, dissolved O_2 concentration) were characterized *in situ* (n = 5 per site over two years) using a YSI 6600 V2-2 Multiparameter Water Quality Sonde (YSI Incorporated) (Table S.3.1). At the sampling sites in both systems, the water column was not stratified, and sampling depths were well oxygenated (>95% O_2 saturation) and within the shallow littoral zone (water column depth was ~3 m at Coldspring Lake and ~1 m at Sunnyside Beach, >1% of PAR irradiance reached the sediment−water interface at both sites). In 2011, diurnal variation in solar UV irradiance was measured at the air−water interface with an Apogee MU-100 UV Sensor (Apogee Instruments). Wind speed and direction (hourly observations) during sampling periods in both years were obtained from the National Climate Data and Information Archive (Environment Canada) for the nearest monitoring station. Solar UV irradiance and wind speed data are summarized in Figure S.3.2.

Slide samplers, consisting of glass microscope slides coated with a collodion polymer membrane to which biological materials adhere upon contact, were prepared and used according to Henk[49] to collect samples from the SML and 0.5 m depth (n = 2 per sampling period) for qualitative microscopic examination of microbial communities (e.g., biofilms). Although collodion is hydrophobic and so may be selective for hydrophobic cells, slide samplers have been demonstrated to collect multispecies biofilms, expected to contain cells of varying hydrophobicity, from natural aquatic environments.[49,50] To sample the SML, horizontally oriented slides were carefully touched to the water surface and then immediately immersed, oriented vertically, and withdrawn, resulting in the adherence of microlayer

materials to the slide.[49] This sampling approach was intended to specifically target the collection of the uppermost surface associated biological materials (e.g., biofilms) present within the SML (i.e., within the top 1 mm of the water column). To sample underneath the SML at 0.5 m depth and avoid contamination from the microlayer, individual slides were placed in sealed sterile 50 mL polypropylene Falcon tubes (BD Biosciences) and lowered by hand to the appropriate depth, at which point the tubes were opened to collect the sample. Following sampling, the back of each slide was wiped dry, and slides were stored in a covered storage box until analysis. To visualize microbial cells, slides were stained with DAPI and viewed at 1000× magnification with a Leica DMRA microscope equipped with an A4 filter cube (excitation 360/40 nm, emission 470/40 nm). At least 50 fields of view were examined per slide.

Sampling of the SML bacterial community (n = 1 at each site in the morning in 2010) was carried out according to Cunliffe et al.[18] using nucleation-track polycarbonate membrane filters (47 mm diameter, 0.1 μm pore size; Isopore; Millipore). This method achieved a sampling depth of ~40 μm.[25,33,51] Select sampling was intentionally carried out in the morning to target the presence of a distinct SML bacterial community, the formation of which is known to be favored under calm (i.e., low energy) conditions,[39,52] which were present at this time of day. For each sample collected, the SML was randomly sampled using five membranes within a ~1 m^2 area, which were collected into a 2 mL screw top tube. To sample the bacterial community at 0.5 m depth, water samples were collected using a Van Dorn sampler (n = 1 at each site in the morning in 2010). The 0.5 m depth water samples were each filtered through cellulose acetate filters (0.2 μm pore size) contained in sterile filter units using a hand pump to produce a vacuum of <10 in Hg. Filters were excised using a

sterile scalpel and placed in sterile Petri dishes. All filters were immediately stored on ice prior to transfer to an −80 °C freezer for longer term storage until DNA isolation.

To collect a sufficient volume of water (~2.5–3 L) from the SML for the analysis of a suite of biogeochemical parameters in a minimal amount of time, a sheet surface sampler with a large surface area was employed to maximize the volume collected per use. The sheet surface sampler consisted of a 110 cm × 110 cm polyethylene sheet with plastic handles affixed at opposite ends on one side (Figure S.3.3). Wood poles were attached to these handles to permit the sampler to be held at arm's length over the edge of a boat. The sampler design was based on similar devices that operate on the principle of SML collection by its adherence to a plastic surface and have been used to collect microlayer samples for geochemical and microbial characterization.[53−55] To sample the SML, the sampler was placed on the water surface and then with a single fluid movement quickly withdrawn and pulled inward, folding the sheet such that a central crease formed via which water adhering to the underside of the sheet drained off under gravity toward the user into a 6 L trace element clean plastic bucket. This was carried out approximately 25–35 times over ~30–40 min to collect a volume of ~2.5–3 L. Based on the volume of water collected per sampling instance and the surface area of the sampler (approach used by Franklin et al.[33]), this method achieved a SML sampling depth of ~60–100 μm. Concurrent with SML sampling with the sheet sampler, a water sample (~2.5–3 L) was collected from 0.5 m depth using a trace element clean Van Dorn sampler.

For each sampling period, bulk water samples from the SML and from 0.5 m depth were collected into 4 L low-density polyethylene carboys (Reliance Products) and

subsequently aliquoted for geochemical and microbial analyses. To minimize organic, nutrient, and trace element contamination, all equipment and containers used for the collection and storage of water samples were soaked in 4% (v/v) HCl for >24 h and then rinsed eight times with ultrapure water (18.2 MΩ cm; Milli-Q; Millipore) before use. Operational field blanks of ultrapure water were also assessed and indicated negligible contamination for each geochemical analyte. For each depth, triplicate aliquots for dissolved trace element analysis (n = 7 sampling periods at each site over two years) were transferred from carboys into acid-washed syringes, sequentially filtered through 0.45- and 0.2-μm syringe filters (Millipore) into 15 mL polypropylene Falcon tubes (BD Biosciences), and acidified to 2% HNO_3 (v/v) using trace element grade concentrated (67%) nitric acid (Fisher Scientific). Triplicate aliquots for total trace element analysis (n = 7 at each site over two years) were directly transferred from carboys into 15 mL Falcon tubes and acidified. Triplicate aliquots for the measurement of microbial abundance and floc size and abundance (n= 7 at each site over two years for total suspended solids (TSS); n = 3 at Coldspring Lake and n= 2 at Sunnyside Beach in 2010 for floc and microbial analyses) were transferred from carboys into 50 mL polypropylene Falcon tubes, and those for microbial analysis were fixed with glutaraldehyde (1% final concentration). The remaining water samples in carboys were retained for dissolved organic carbon (DOC, <0.45 μm) (2011 only, n = 4 at Coldspring Lake and n = 5 at Sunnyside Beach) and nutrient analyses (n = 7 at each site over two years). At Sunnyside Beach in 2010, additional water was transferred from carboys into 120 mL glass bottles previously cleaned with 10% H_2SO_4 for DOC analysis (n = 2) as well as 500 mL Teflon containers for particulate organic carbon and nitrogen (POC and PON) analysis (n = 2). With the exception of aliquots for POC/PON and floc size and abundance, which were

frozen at −20 °C in the dark until analysis, all aliquots to be analyzed were stored at 4 °C in the dark until analysis.

3.3.3 Environmental Bacterial Community Composition

Total community DNA was isolated from the biological material collected on filters using the PowerSoil DNA Isolation Kit (MO Bio Laboratories) according to the manufacturer's instructions. Bacterial 16S rRNA genes were selectively amplified from total community DNA by PCR using the 27F/1492R primer set according to Zwart et al.[56] and then cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). At least 50 clones containing inserts were sequenced (>700 nucleotides) for each sample (MOBIX Lab, McMaster University, Hamilton, Ontario, Canada). Although a relatively small number of sequences were analyzed for each sample, these numbers were sufficient to accurately represent the composition of environmental bacterial communities at higher taxonomic levels (i.e., phyla and classes of the phylum *Proteobacteria*)[57,58] and permit statistically significant differences between communities at these levels to be identified. These sequences were published in the GenBank database under the accession numbers KM031105 to KM031329. The closest database matches to each sequence were obtained using BLAST (http://blast.ncbi.nlm.nih.gov/). The Library Compare online analysis tool, available as part of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/), was used to identify significant taxonomic differences between clone libraries.[59] For this analysis, phylotypes were defined at a 97% sequence similarity level.

3.3.4 Floc Analyses

To quantify the abundance and size distribution of suspended flocs, water samples of known volume were passed through polycarbonate membrane filters (0.1 μm pore size) by vacuum filtration and imaged using a Leica DMRA microscope (Leica Microsystems). Bright field images were captured using OpenLab software (version 5.1) (PerkinElmer). Images of at least 35 fields of view were captured at 1000× magnification for Sunnyside Beach samples and at 100× magnification for Coldspring Lake samples. Floc abundance and size (i.e., area based on a two-dimensional top-down view) were measured using ImageJ software (version 1.43) (http://rsb.info.nih.gov/ij/).[60]

3.3.5 Geochemical Analyses

Dissolved (<0.2 μm) and total soluble Fe (Fe(II) + Fe(III)) concentrations were measured in triplicate previously acidified water samples using the colorimetric FerroVer method based on 1,10-phenanthroline complexation (Hach Company). Dissolved (<0.2 μm) and total trace element (Ba, Cu, Mn, Ni, Sr) concentrations were quantified in triplicate, previously acidified water samples by inductively coupled plasma mass spectrometry (Sciex Elan 6100; PerkinElmer). These elements were chosen as they include both conservative (Ba and Sr) and nonconservative (Cu and Ni) elements with respect to their relative affinity for organics and Fe. Field and procedural blanks demonstrated that there was negligible contamination (<5%) for all analyzed trace elements. Total soluble reactive phosphate (PO_4^{3-}), nitrate (NO_3^-–N), and ammonia (NH_3–N) were measured in triplicate using spectrophotometric Hach methods (PhosVer 3 ascorbic acid method, cadmium reduction method, and Nessler method, respectively) as per the manufacturer's instructions. Where

reported, trace element and nutrient concentration values are given as mean ± standard error.

For 2010 water samples, DOC (<0.45 μm) concentrations were measured in triplicate using a UV-persulfate TOC analyzer according to methods at the National Laboratory for Environmental Testing (NLET).[61] For 2011 water samples, DOC concentrations were measured in triplicate using a spectrophotometric method based on absorption at 330 nm, adjusted for Fe interference.[62] POC and PON concentrations were measured by filtering water samples of known volume onto precombusted glass fiber filters, rinsing the collected particulate matter with 0.3% H_2SO_4 (v/v) to remove inorganic carbon and nitrogen, and then measuring CO_2 and N_2 released by complete oxidation, according to NLET methods.[61] TSS concentrations were measured in triplicate using a photometric method based on the absorbance of a homogenized sample at 810 nm (Hach Company). Where reported, POC and PON concentrations were mean ± standard error.

3.3.6 Enumeration of Prokaryotes and Autotrophic Picoplankton

Prokaryotes in triplicate water samples were directly enumerated by filtering water samples of known volume onto black polycarbonate filters (0.1 μm pore size, Isopore; Millipore), staining with DAPI, and visualizing at 1000× magnification with a Leica DMRA microscope equipped with an A4 filter cube (excitation 360/40 nm, emission 470/40 nm) according to Kepner and Pratt.[63] Following the same procedure, without DAPI staining, autofluorescent phycoerythrin-containing picocyanobacteria were enumerated by epifluorescence miscroscopy using a GFP filter cube (excitation 470/40 nm, emission

525/50 nm).[64] For each cell type, at least 400 cells in more than 20 microscopic fields per filter were counted to achieve an accurate measure of cell abundance.

3.3.7 Statistical Analysis

Statistical analysis was carried out using StatPlus:mac LE software (AnalystSoft, http://www.analystsoft.com/). Statistical comparisons between systems and between water depths were each made by one-way ANOVA, followed by Tukey's post hoc test. Simple linear regression (Pearson's correlation coefficient) was used to determine the relationship between pairs of physicochemical and geochemical parameters. *P* values <0.05 were considered significant.

3.4 Results and Discussion

Although SML geochemistry was system-dependent, showing significantly higher ($P < 0.05$) concentrations of DOC, PO_4^{3-}, Fe, and Mn but lower ($P < 0.05$) concentrations of Ba, Cu, Ni, and Sr in Coldspring Lake relative to Sunnyside Beach (Table S.3.1), thin yet extensive biofilms were observed in SML samples from both systems, which were not present in either matching 0.5 m depth samples (Figure 3.1). Similar SML biofilms have been observed in other freshwater systems,[30,49,65] identifying the importance of surface tension stabilization creating a distinct pseudosurface at the air–water interface for microbial colonization and biofilm development. The SML of the ocean has similarly been proposed to be a gelatinous biofilm with an extracellular matrix consisting largely of coagulated organic matter produced by phytoplankton.[18,30]

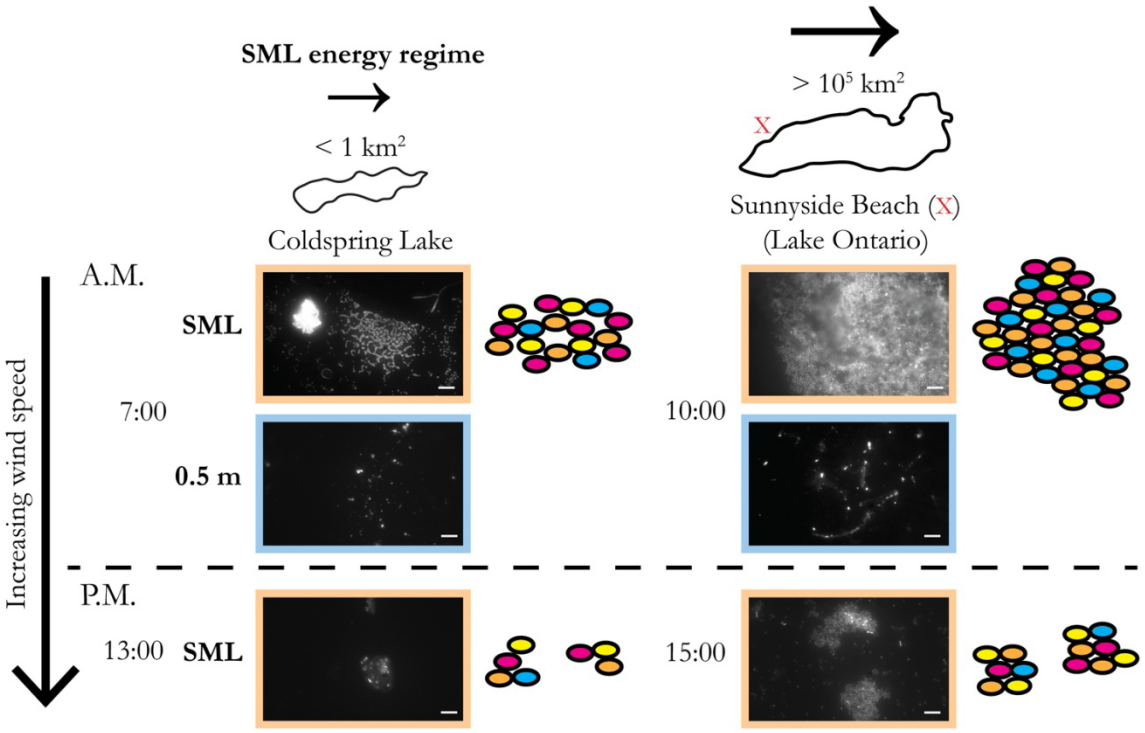

Figure 3.1 Distinct surface microlayer (SML) biofilms observed at Coldspring Lake and Sunnyside Beach in the morning and afternoon. Bar represents 10 μm.

Bacterial 16S rRNA gene clone libraries were generated for a single set of water samples from the SML and at 0.5 m depth for each system in the morning in 2010, under relatively quiescent conditions that appeared to favor SML biofilm formation (Figure 3.1). The majority (>90%) of library sequence matches from both systems and depths were to bacteria from oxic freshwater environments. However, several significant ($P < 0.05$) differences at the phylum and class levels were found between the SML and 0.5 m depth bacterial communities in each system (Figure 3.2). *Actinobacteria*, a major phylum in freshwater environments, were relatively depleted in both SML clone libraries (Figure 3.2). In freshwater lakes, *Actinobacteria* are typically present as relatively slow growing single cells

poorly suited to biofilm environments,[66] consistent with their reduced presence in SML bacterial communities in this study. In contrast to *Actinobacteria*, the beta and gamma classes of the phylum *Proteobacteria* were enriched in clone libraries from the SML relative to 0.5 m depth at Coldspring Lake and Sunnyside Beach, respectively (Figure 3.2). Both of these classes include fast-growing heterotrophic bacteria and are thus often associated with biofilms and other sites of increased nutrient availability in freshwater lakes,[66] which are conditions that can be found in the SML. Further, *Gammaproteobacteria* are typically poorly represented in freshwater bacterial communities,[66] highlighting the distinctiveness of their enrichment in the Sunnyside Beach SML clone library. SML *Betaproteobacteria* populations have been observed to closely resemble airborne populations,[36,38] and *Gammaproteobacteria* have been found in dust inputs to surface waters,[67,68] implicating deposition from the atmosphere, another extreme environment, as a possible source of distinct and extreme-adapted SML bacteria. The relatively increased representation of *Betaproteobacteria* and decreased representation of *Actinobacteria* in bacterial communities from the SML compared to underlying waters has also been observed across several alpine lakes,[36–38] indicating differences in the ability of these bacterial groups to colonize the SML. However, other bacterial groups did not show such distinct SML versus underlying water column trends. *Cyanobacteria* were detected only in the 0.5 m depth clone library from Coldspring Lake and only in the SML clone library at Sunnyside Beach (Figure 3.2).

All *Cyanobacteria* sequences in the Sunnyside Beach SML library most closely matched (98% sequence identity) a filamentous bacterium, *Phormidium autumnale* (GenBank accession number FJ866617), that was the principal constituent of microbial mats in Lake Huron, another of the Laurentian Great Lakes.[69] The prominence of this bacterium in microbial

mats indicates that it contributed to the formation of SML biofilms at Sunnyside Beach. In contrast, the majority of *Cyanobacteria* sequences in the 0.5 m depth clone library from Coldspring Lake mostly closely matched (>98% sequence similarity) freshwater members of the picocyanobacteria genera *Cyanobium* and *Synechococcus*. These genera typically occur as solitary cells,[64] suggesting that their absence from the corresponding SML library reflected the relative predominance of biofilm-based bacteria in the microlayer. Thus, it appears that the presence of distinct SML biofilms was a key determinant of the differing composition of bacterial communities between the SML and underlying waters within each system, regardless of the system specificity of community composition. Biofilm formation can confer resistance to UV radiation[70] and toxic metals[71] and so may be employed by SML bacteria to better withstand elevated exposure to these detrimental factors at the air–water interface.

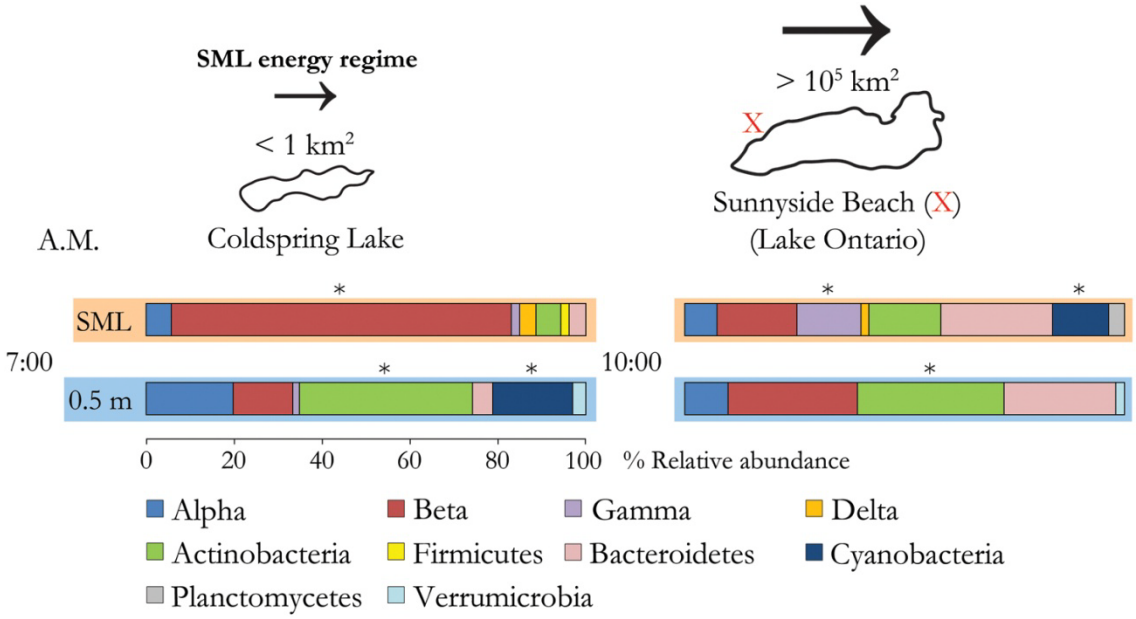

Figure 3.2 Relative abundance of phyla and *Proteobacteria* classes (alpha, beta, gamma, and delta) detected in bacterial 16S rRNA gene clone libraries from the surface microlayer (SML) and 0.5 m depth at Coldspring Lake and Sunnyside Beach in the morning. *Significantly higher ($P < 0.05$) within a system between water depths.

For both systems, SML clone libraries included close (>97% sequence identity) matches to known As-, Fe-, and S-metabolizing bacterial isolates or enrichments (Table S.3.2). However, matches to these metabolisms were absent from the corresponding libraries from 0.5 m depth indicating a characteristically greater functional diversity within SML communities. They also point to the occurrence of anaerobic metabolisms within the SML bacterial community and thus the presence of microenvironments capable of hosting such metabolisms in the microlayer. Biofilms commonly exhibit internal gradients of pH, O_2, and various bacterial substrates.[72–74] The presence of a compositionally distinct SML bacterial community that consistently differs from the underlying water column reveals the potential for differential biogeochemical cycling within this zone.

In addition to differences in the bacterial communities associated with the SML, at Sunnyside Beach and, depending on the time of day, Coldspring Lake, the abundance of flocs (>50 μm^2) was greater in the SML compared to 0.5 m depth (Figure 3.3) indicating floc collection and/or generation directly within this zone. However, the presence of larger and more complex biofilms in the SML at Sunnyside Beach (Figure 3.1) was correlated to the abundance of flocs (>50 μm^2) in the microlayer (Figure 3.3). This phenomenon was not observed in the underlying water, suggesting a more active SML role in floc generation. Surprisingly, although SML biofilms were larger and the number of flocs (>50 μm^2) in the microlayer was an order of magnitude higher at Sunnyside Beach (Figure 3.3), numbers of prokaryotes and phycoerythrin-containing picocyanobacteria (Figure S.3.4) and mean whole-day concentrations of DOC, Fe, and PO_4^{3-} (Table S.3.1), important floc components and expected facilitators of floc generation[5,29] were higher ($P < 0.05$ for geochemical parameters, cell numbers not tested for statistical significance due to small sample sizes but differed by an order of magnitude between systems) at Coldspring Lake, indicating that absolute concentrations of biofilm components are not key predictors of SML biofilm or floc size.

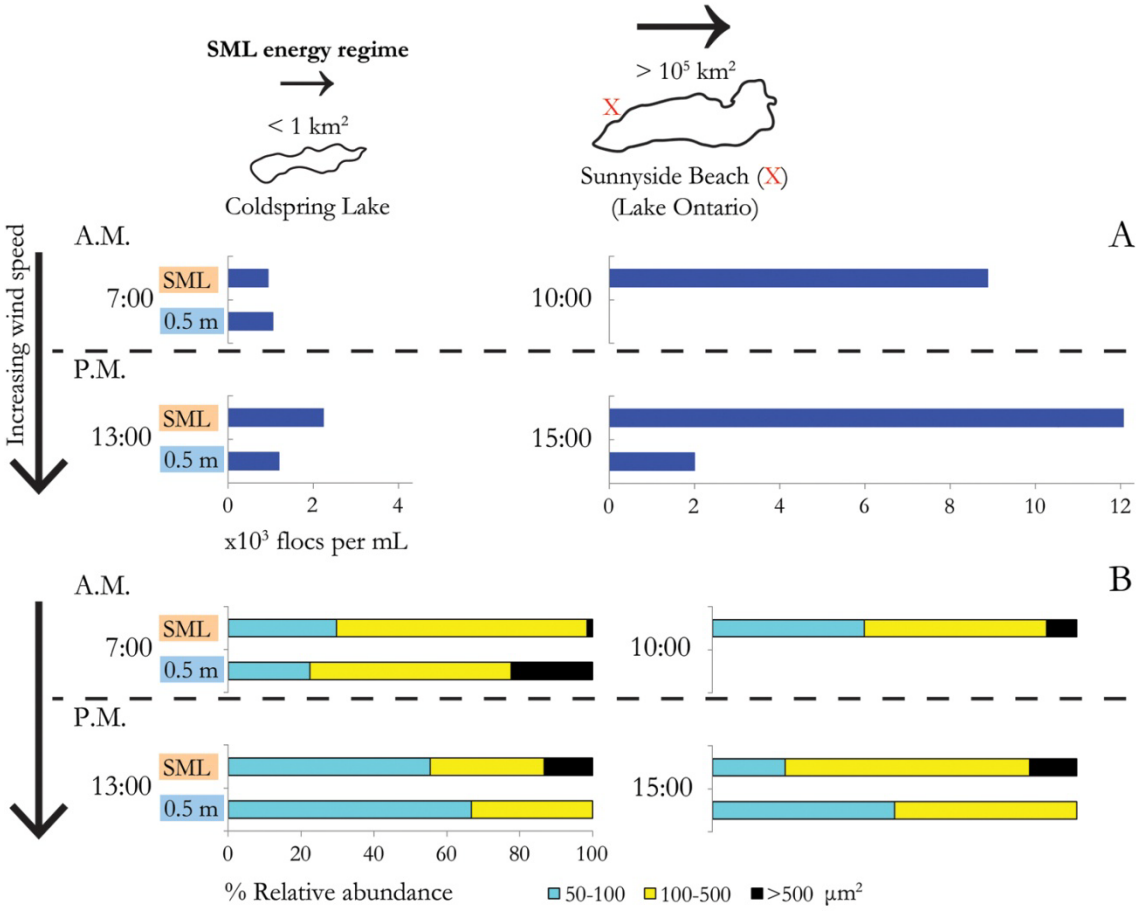

Figure 3.3 (A) Abundance of flocs (>50 µm²) and (B) relative distribution of floc size fractions in the surface microlayer (SML) and at 0.5 m depth at Coldspring Lake and Sunnyside Beach in the morning and afternoon.

However, in each system, increasing wind speeds and solar UV radiation exposure (i.e., increasing system energy) from morning to afternoon (Figure S.3.2) were associated with a decrease in SML biofilm size and an increase in the abundance of flocs (>50 µm^2) as well as the relative proportion of the largest flocs (>500 µm^2) (Figure 3.3). In addition to the potential contribution of photosynthetic floc inhabitants to increasing floc size over diurnal time scales, the link between decreasing biofilm size and increasing floc size and abundance implicates physical forces in SML-based floc generation. The results indicate the key

influence of a diurnally variable system energy regime on SML biofilm formation and subsequent floc generation. A direct link between physical forces and floc generation from SML organic materials was previously reported by Wheeler,[75] who observed that experimentally induced increases in surface pressures, otherwise driven by wind or wave action *in situ*, resulted in the generation of particulate matter from microlayer organic material. Results from Sunnyside Beach (Coldspring Lake not available) for diurnal organic carbon trends are consistent with increasing energy regime impacts associated with (1) SML collection of floc building bloc (organic) materials and (2) higher energy regime associated biofilm break down. Mean whole-day concentrations of POC and PON (g/g TSS) were an order of magnitude higher in the microlayer relative to 0.5 m depth (0.314 ± 0.016 vs 0.065 ± 0.002 for POC and 0.035 ± 0.001 vs 0.008 ± 0.001 for PON, respectively), identifying the accumulation of organic matter in the SML through *in situ* biofilm formation and/or upward transport of organic matter from the underlying water column. However, as wind speed increased from morning to afternoon, a significant ($P < 0.05$) increase in SML [DOC] (3.4 ± 0.1 mg/L to 4.1 ± 0.1 mg/L) and significant ($P < 0.05$) decrease in [POC] (0.37 ± 0.02 g/g TSS to 0.26 ± 0.02 g/g TSS) occurred, pointing to the wind-driven breakdown of SML biofilms. Emphasizing this breakdown, the south/west wind direction during sampling at Sunnyside Beach means that organic particulates were likely transported by wind-driven currents toward the sampling area from offshore and from adjacent littoral regions, yet SML POC concentrations still decreased diurnally. These results illustrate the highly temporally dynamic, differential impacts of physical forces stemming from wind speed on SML microbial structuring, as they were sufficient to break up the thin microlayer biofilms, while also promoting their aggregation into larger flocs. The impact of increasing system energy

regime magnitude is not simply to break SML biofilms apart; energetic disturbances appear to be required for the generation of floc from SML biofilms. Thus, system-dependent energy regime intensity, as opposed to the relative availability of structural building blocks (i.e., system biogeochemistry), appears to be a dominant control on the extent of SML biofilm and floc development.

Floc generation from biofilms in the SML and subsequent settling out of the microlayer provides an important mechanism for the accumulation and transport of atmospherically introduced or microlayer-incubated microorganisms, nutrients, and contaminants to the underlying water column and bed sediments.[46] Results here identify that SML-derived flocs, biogeochemically reflective of the different conditions occurring within this microenvironment, will differentially impact biogeochemical cycling relative to that occurring within the bulk oxic water column. As with biofilms, SML flocs can provide sheltered microenvironments for microorganisms potentially inhibited by the otherwise extreme conditions present at the air–water interface.[76] The presence of larger (>500 μm^2) flocs and a largely heterotrophic bacterial community in the SML points to the development of floc-based O_2 gradients as a result of localized heterotrophic oxygen consumption.[5,8,77] O_2-depleted pockets within SML flocs would be suitable for hosting anaerobic bacterial metabolisms such as Fe or S reduction that would be otherwise inhibited by bulk oxic conditions.[78,79] Underscoring the selective biogeochemical cycling that occurs within the SML, and consistent with the occurrence of higher concentrations of organics and Fe in the SML, enrichments factors (SML [element]/0.5 m [element]) for reactive trace elements Cu and Ni were typically >1, while those for conservative elements Sr and Ba were not (Figure S.3.5).

The observed differences identified here between the SML and 0.5 m depth with respect to biofilm-associated geochemistry, microbial community structure and abundance, as well as the physical generation of floc that extends SML effects into the bulk water column highlight the small distances over which processes important to system-level biogeochemical cycling can vary. Even under the higher energy regime that impacted a shallower water column (~1 m depth) at Sunnyside Beach, relative to Coldspring Lake (~3 m depth), a positive influence of wind speed on TSS concentration was found at 0.5 m depth ($R^2 = 0.76$, $P < 0.05$) but not in the microlayer ($R^2 = 0.23$) (Figure S.3.6). This finding identifies that wind-driven bed sediment resuspension, a fundamental mechanism of biogeochemical cycling in freshwater lakes,[14,80,81] is a major source of flocs in the waters underlying the SML but not in the microlayer itself, underscoring the distinctiveness of the SML microbial community and its influence on microlayer geochemistry. In keeping with the selective impact of bed sediment resuspension at Sunnyside Beach on the composition of subsurface flocs and its disconnect from the SML, TSS concentrations at 0.5 m depth were positively correlated with concentrations of particulate (>0.2 μm) Fe ($R^2 = 0.87$, $P < 0.05$) and DOC ($R^2 = 0.81$, $P < 0.05$), but similar correlations were not found for the microlayer.

Results here demonstrate that while SML geochemistry largely reflects water column bulk characteristics, the microlayer provides a distinct habitat for selective biofilm producing bacterial communities. These biofilms are precursors from which larger flocs, retaining an SML biogeochemical signature, were generated. The SML was impacted by exposure to diurnally increasing wind and sunlight, with system-specific energy regime magnitude, as opposed to water column biogeochemistry, directly determining floc size and abundance. The presence of SML biofilms and flocs was associated with the enrichment of trace

elements in the microlayer and potentially supplied microhabitats for otherwise unfavorable metabolic groups within the bulk oxic water column. The generation and settling out of flocs from the SML provides a means of concentrating and disseminating microorganisms, nutrients, and contaminants to the underlying bulk water column.

3.5 Supporting Information

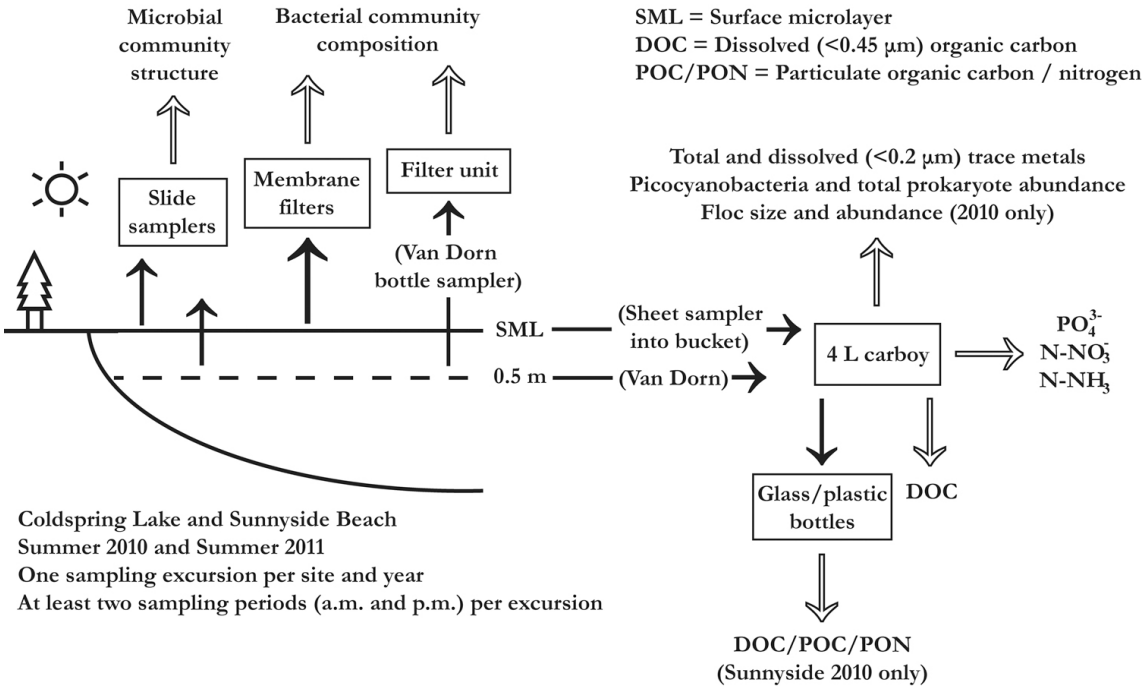

Figure S.3.1 Schematic of water sample collection and aliquoting for geochemical and microbial analysis.

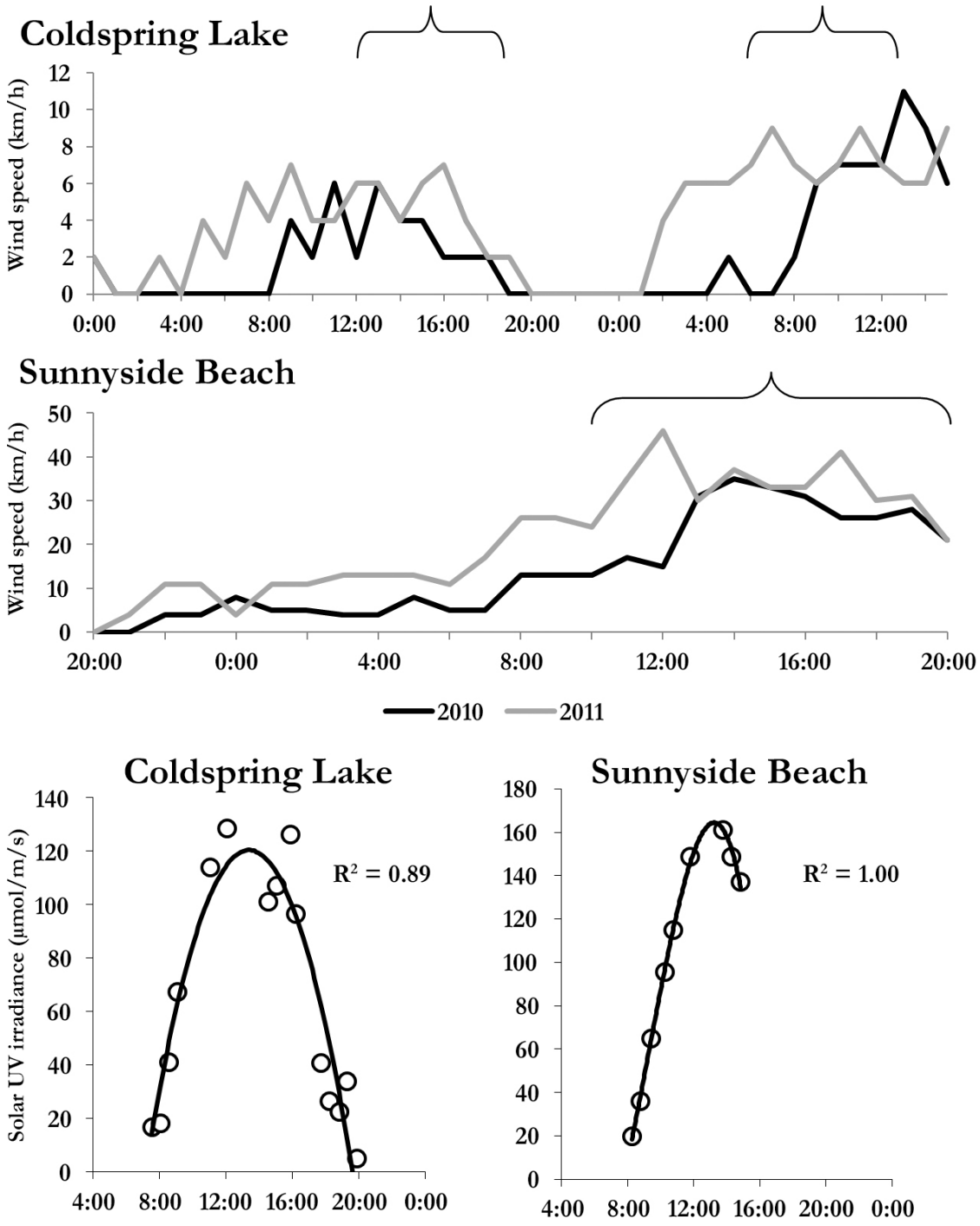

Figure S.3.2 Wind speeds in 2010 and 2011, and solar UV irradiance in 2011, at Coldspring Lake and Sunnyside Beach during sampling. Brackets indicate sampling periods.

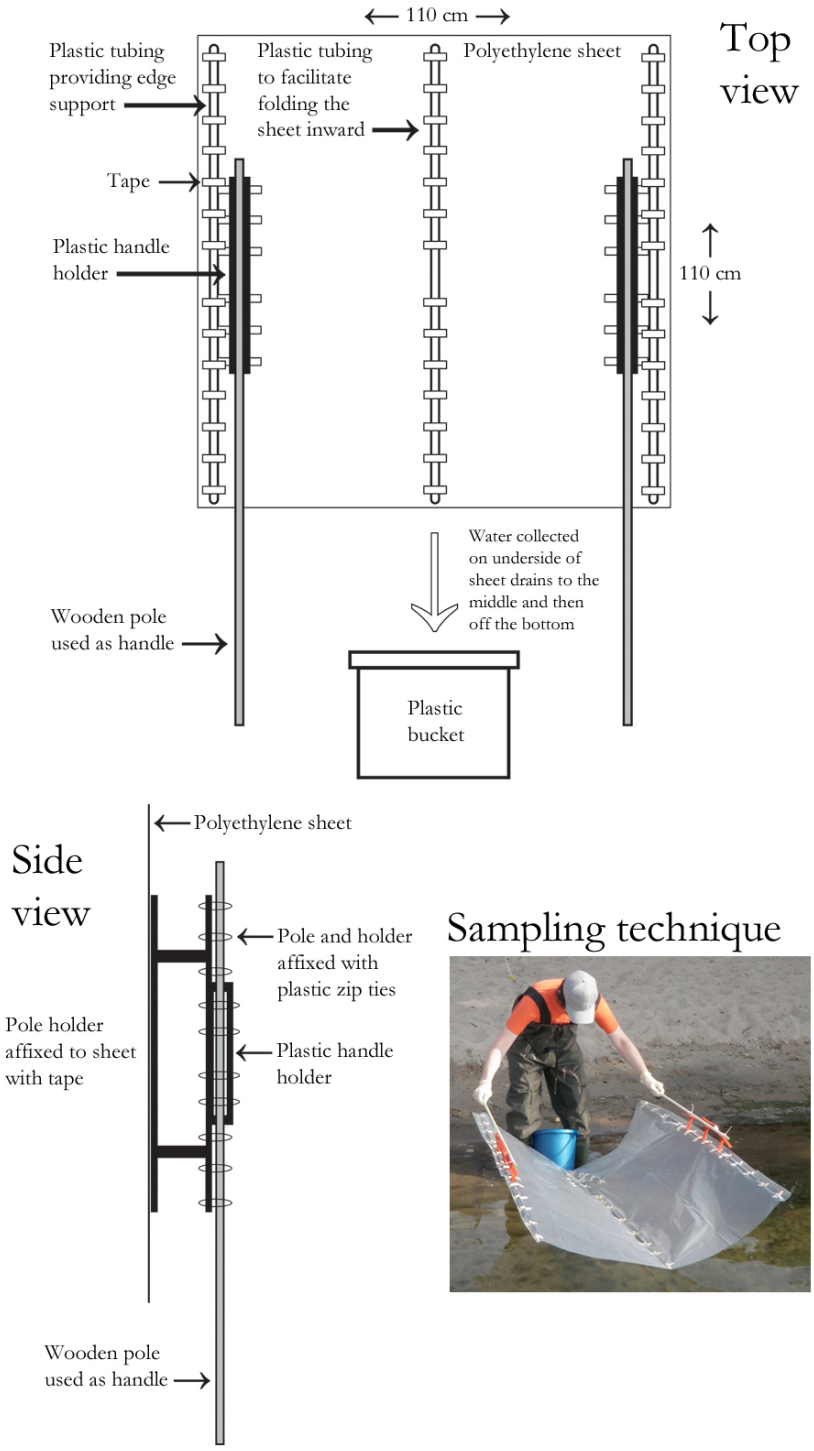

Figure S.3.3 Surface microlayer sheet sampler.

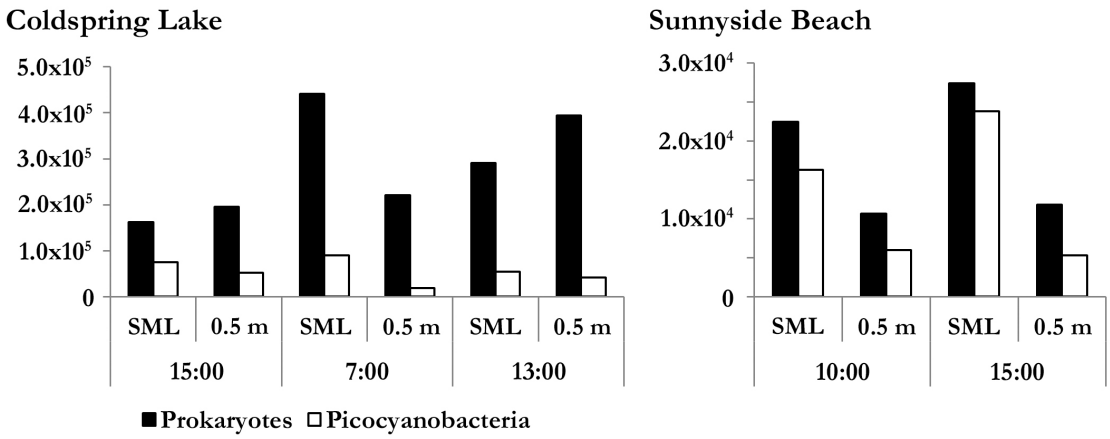

Figure S.3.4 Prokaryote and phycoerythrin-containing picocyanobacteria cell abundance (cells per mL) at Coldspring Lake and Sunnyside Beach in 2010. SML = Surface microlayer.

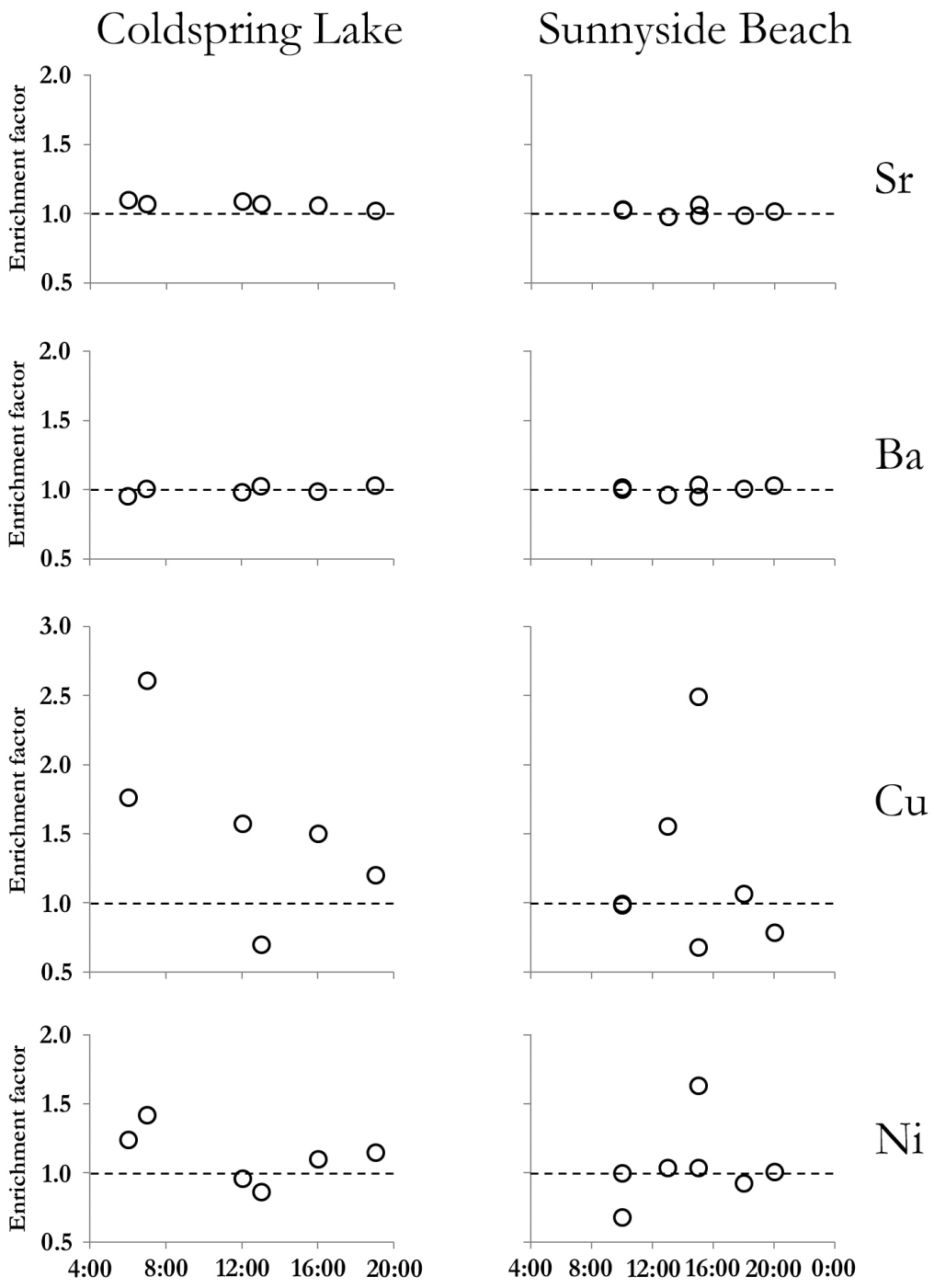

Figure S.3.5 Diurnal surface microlayer (SML) enrichment factors (SML [element] / 0.5 m depth [element]) for dissolved (<0.2 μm) Sr, Ba, Cu, and Ni at Coldspring Lake and Sunnyside Beach over consecutive summers.

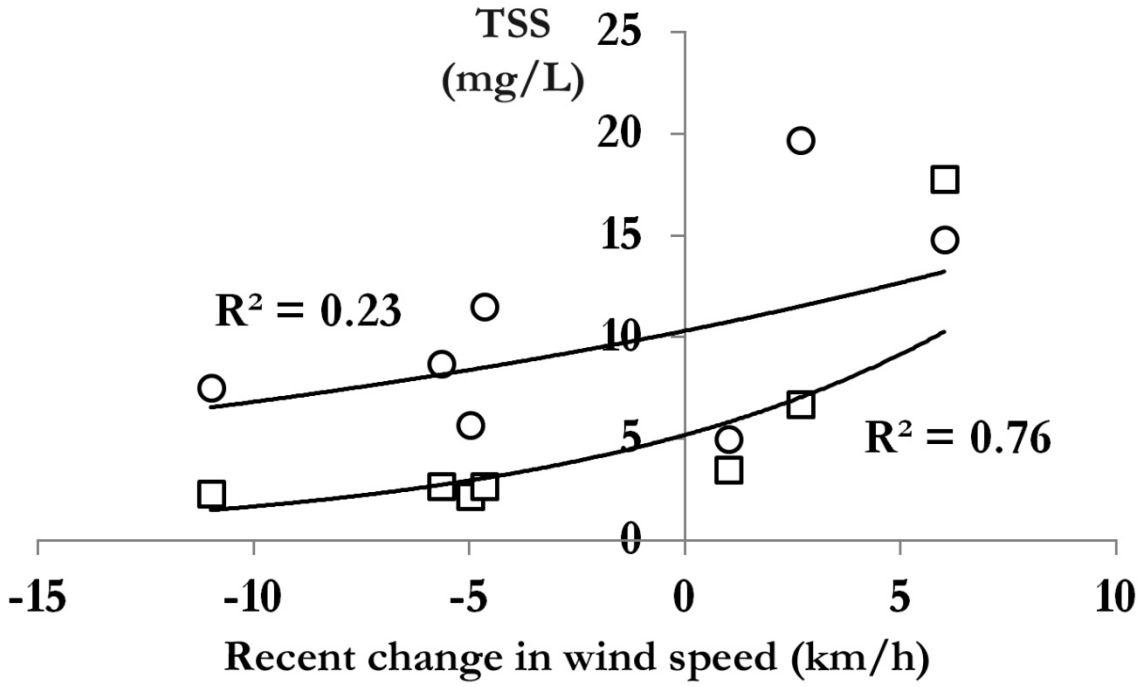

Figure S.3.6 Relationship between concentration of total suspended solids (TSS) and recent change in wind speed (previous three hour average speed – speed at time of sampling) in the surface microlayer (circles) and at 0.5 m depth (squares) at Sunnyside Beach over consecutive summers.

Table S.3.1 Mean (± standard error) whole-day (i.e. inclusive of all samples collected throughout the day) physicochemical parameters and concentrations of total suspended solids (TSS), dissolved organic carbon (DOC), nutrients, and trace elements for the surface microlayer (SML) and 0.5 m depth of Sunnyside Beach and Coldspring Lake, sampled in consecutive summers (2010 and 2011).

Parameter	**Coldspring Lake**		**Sunnyside Beach**	
	SML	**0.5 m depth**	**SML**	**0.5 m depth**
Temperature (°C)	24.9 ± 0.4#	24.9 ± 0.4#	22.1 ± 0.7	21.9 ± 0.7
pH	6.9 ± 0.1#	7.0 ± 0.1#	8.4 ± 0.1	8.4 ± 0.1
O_2 (% saturation)	97.1 ± 1.0#	96.9 ± 1.0#	106.2 ± 2.1	106.8 ± 2.3
TSS (mg/L)	4.6 ± 0.4#	3.2 ± 0.3	10.3 ± 2.1*	5.3 ± 1.9
DOC (mg/L)	4.2 ± 0.1#*	4.5 ± 0.1#	3.2 ± 0.3*	2.5 ± 0.1
PO_4^{3-} (total) (mg/L)	0.07 ± 0.01#	0.09 ± 0.02#	0.04 ± 0.01	0.03 ± 0.01
NO_3^--N (total) (mg/L)	0.8 ± 0.3	0.8 ± 0.2	1.2 ± 0.1	1.2 ± 0.2
NH_3-N (total) (mg/L)	0.16 ± 0.01#*	0.12 ± 0.01#	0.40 ± 0.01*	0.06 ± 0.02
Fe (total) (μM)	3.6 ± 1.0#	3.0 ± 0.6#	1.6 ± 0.7	1.2 ± 0.3
Fe (<0.2 μm) (μM)	1.8 ± 0.4#	1.5 ± 0.4#	0.6 ± 0.1	0.5 ± 0.1
Mn (total) (μM)	0.38 ± 0.01#	0.37 ± 0.01#	0.23 ± 0.05	0.21 ± 0.06
Mn (<0.2 μm) (μM)	0.09 ± 0.02	0.10 ± 0.03	0.06 ± 0.01	0.05 ± 0.02
Cu (total) (μM)	0.021 ± 0.002#	0.021 ± 0.004#	0.067 ± 0.08	0.057 ± 0.06
Cu (<0.2 μm) (μM)	0.021 ± 0.001	0.017 ± 0.002	0.025 ± 0.007	0.021 ± 0.001
Ni (total) (μM)	0.013 ± 0.001#	0.009 ± 0.001#	0.038 ± 0.005	0.038 ± 0.004
Ni (<0.2 μm) (μM)	0.011 ± 0.002#	0.009 ± 0.001#	0.035 ± 0.003	0.036 ± 0.003
Ba (total) (μM)	0.107 ± 0.018#	0.093 ± 0.004#	0.192 ± 0.004	0.187 ± 0.007
Ba (<0.2 μm) (μM)	0.092 ± 0.004#	0.084 ± 0.003#	0.183 ± 0.002	0.181 ± 0.003
Sr (total) (μM)	0.26 ± 0.01#	0.28 ± 0.02#	2.08 ± 0.02	2.05 ± 0.02
Sr (<0.2 μm) (μM)	0.26 ± 0.01#	0.26 ± 0.01#	2.04 ± 0.03	2.04 ± 0.02

Significantly different ($P < 0.05$) between systems at the same water depth.

* Significantly different ($P < 0.05$) within a system between water depths.

For physicochemical parameters, n=5 per depth for each system. For DOC, n=4 at Coldspring Lake and n=5 at Sunnyside Beach per depth (2011 only). For all other parameters, n=7 per depth for each system.

Table S.3.2 Close (>97% sequence identity) matches to isolates or constituents of enrichments or environmental communities that metabolize As, Fe, or S in surface microlayer bacterial 16S rRNA gene clone libraries from Sunnyside Beach and Coldspring Lake in 2010.

Site	Sequence matches (Accession no., % identity)
Sunnyside Beach	• Anaerobic dissimilatory Fe(III)-reducing enrichment from arsenic contaminated paddy soil (FJ269052, 99%) • Anaerobic denitrifying Fe(II)-oxidizing bacterium from freshwater mud (U51102, 98%) • Uncultured *Desulfobulbus* sp. (anaerobic sulfate- and Fe(III)-reducing bacterium) from brackish river sediment (DQ831533, 99%) • Uncultured bacterium from Frasassi cave system sulfidic spring biofilm (JF747985, 98%)
Coldspring Lake	• *Leptothrix cholodnii* SP-6 (Fe(II)-oxidizing bacterium) (NR_074623, 98%) • Uncultured bacterium from arsenite-oxidizing biofilm in a geothermally impacted stream (AY168738, 99%) • *Limnobacter thiooxidans* CS-K2 (aerobic thiosulfate-oxidizing bacterium) from freshwater lake sediment (NR_025421, 97%)

3.6 Acknowledgements

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3.7 References

(1) Tonolla, M.; Peduzzi, S.; Hahn, D.; Peduzzi, R. Spatio-temporal distribution of phototrophic sulfur bacteria in the chemocline of meromictic Lake Cadagno (Switzerland). FEMS Microbiol. Ecol. 2003, 43 (1), 89−98, DOI: 10.1111/j.1574-6941.2003.tb01048.x.

(2) Chapin, B. R. K.; DeNoyelles, F.; Graham, D. W.; Smith, V. H. A deep maximum of green sulphur bacteria ('*Chlorochromatium aggregatum*') in a strongly stratified reservoir. Freshwater Biol. 2004, 49 (10), 1337−1354, DOI: 10.1111/j.1365-2427.2004.01270.x.

(3) Selig, U.; Hübener, T.; Heerkloss, R.; Schubert, H. Vertical gradient of nutrients in two dimictic lakes − influence of phototrophic sulfur bacteria on nutrient balance. Aquat. Sci. 2004, 66 (3), 247−256, DOI: 10.1007/s00027-004-0684-y.

(4) Jones, C.; Crowe, S. A.; Sturm, A.; Leslie, K. L.; MacLean, L. C. W.; Katsev, S.; Henny, C.; Fowle, D. A.; Canfield, D. E. Biogeochemistry of manganese in Lake Matano, Indonesia. Biogeosciences Discuss. 2011, 8, 4063−4106, DOI: 10.5194/bgd-8-4063-2011.

(5) Simon, M.; Grossart, H. P.; Schweitzer, B.; Ploug, H. Microbial ecology of organic aggregates in aquatic ecosystems. Aquat. Microb. Ecol. 2002, 28 (2), 175−211, DOI: 10.3354/ame028175.

(6) Arnous, M. B.; Courcol, N.; Carrias, J. F. The significance of transparent exopolymeric particles in the vertical distribution of bacteria and heterotrophic nanoflagellates in Lake Pavin. Aquat. Sci. 2010, 72 (2), 245−253, DOI: 10.1007/s00027-010-0127-x.

(7) Kalenak, D.; Boss, E.; Effler, S. W. Inherent optical properties of suspended particulates in four temperate lakes: application of *in situ* spectroscopy. Hydrobiologia 2013, 713 (1), 127−148, DOI: 10.1007/s10750-013-1498-5.

(8) Liss, S. N.; Droppo, I. G.; Flannigan, D. T.; Leppard, G. G. Floc architecture in wastewater and natural riverine systems. Environ. Sci. Technol. 1996, 30 (2), 680−686, DOI: 10.1021/es950426r.

(9) Droppo, I. G. Rethinking what constitutes suspended sediment. Hydrol. Processes 2001, 15 (9), 1551−1564, DOI: 10.1002/hyp.228.

(10) Patrolecco, L.; Ademollo, N.; Capri, S.; Pagnotta, R.; Polesello, S. Occurrence of priority hazardous PAHs in water, suspended particulate matter, sediment and common eels (*Anguilla anguilla*) in the urban stretch of the River Tiber (Italy). Chemosphere 2010, 81 (11), 1386−1392, DOI: 10.1016/j.chemosphere.2010.09.027.

(11) Elliott, A. V. C.; Plach, J. M.; Droppo, I. G.; Warren, L. A. Comparative floc-bed sediment trace element partitioning across variably contaminated aquatic ecosystems. Environ. Sci. Technol. 2012, 46 (1), 209−216, DOI: 10.1021/es202221u.

(12) Hua, X.; Dong, D.; Liu, L.; Gao, M.; Liang, D. Comparison of trace metal adsorption onto different solid materials and their chemical components in a natural aquatic environment. Appl. Geochem. 2012, 27 (5), 1005−1012, DOI: 10.1016/j.apgeochem.2012.01.021.

(13) Droppo, I. G. Biofilm structure and bed stability of five contrasting freshwater sediments. Mar. Freshwater Res. 2009, 60 (7), 690−699, DOI: 10.1071/MF08019.

(14) Plach, J. M.; Elliott, A. V. C.; Droppo, I. G.; Warren, L. A. Physical and ecological controls on freshwater floc trace metal dynamics. Environ. Sci. Technol. 2011, 45 (6), 2157−2164, DOI: 10.1021/es1031745.

(15) Hardy, J. T. The sea surface microlayer: biology, chemistry and anthropogenic enrichment. Prog. Oceanogr. 1982, 11 (4), 307−328, DOI: 10.1016/0079-6611(82)90001-5.

(16) Maki, J. S.; Hermansson, M. The dynamics of surface microlayers in aquatic environments. In The Biology of Particles in Aquatic Systems; Wotton, R. S., Ed.; CRC Press: Boca Raton, FL, 1994; pp 161−182.

(17) Gladyshev, M. I. Biophysics of the Surface Microlayer of Aquatic Ecosystems; IWA Publishing: London, U.K., 2002.

(18) Cunliffe, M.; Upstill-Goddard, R. C.; Murrell, J. C. Microbiology of aquatic surface microlayers. FEMS Microbiol. Rev. 2011, 35 (2), 233−346, DOI: 10.1111/j.1574-6976.2010.00246.x.

(19) Cunliffe, M.; Engel, A.; Frka, A.; Gašparović, B.; Guitart, C.; Murrell, J. C.; Salter, M.; Stolle, C.; Upstill-Goddard, R.; Wurl, O. Sea surface microlayers: A unified physicochemical and biological perspective of the air−ocean interface. Progr. Oceanogr. 2013, 109, 104−116, DOI: 10.1016/j.pocean.2012.08.004.

(20) Williams, P. M.; Carlucci, A. F.; Henrichs, S. M.; Van Vleet, E. S.; Horrigan, S. G.; Reid, F. M. H.; Robertson, K. J. Chemical and microbiological studies of sea-surface films in the southern Gulf of California and off the west coast of Baja California. Mar. Chem. 1986, 19 (1), 17−98, DOI: 10.1016/0304-4203(86)90033-2.

(21) Södergren, A. Role of aquatic surface microlayer in the dynamics of nutrients and organic compounds in lakes, with implications for their ecotones. Hydrobiologia 1993, 251 (1−3), 217−225, DOI: 10.1007/BF00007181.

(22) Münster, U.; Heikkinen, E.; Knulst, J. Nutrient composition, microbial biomass and activity at the air-water interface of small boreal forest lakes. Hydrobiologia 1997, 363 (1−3), 261−270, DOI: 10.1023/A:1003106713091.

(23) Reinthaler, T.; Sintes, E.; Herndl, G. J. Dissolved organic matter and bacterial production and respiration in the sea-surface microlayer of the open Atlantic and the western Mediterranean Sea. Limnol. Oceanogr. 2008, 53 (1), 122−136, DOI: 10.4319/lo.2008.53.1.0122.

(24) Baastrup-Spohr, L.; Staehr, P. A. Surface microlayers on temperate lowland lakes. Hydrobiologia 2009, 625 (1), 43−59, DOI: 10.1007/s10750-008-9695-3.

(25) Crawford, R. L.; Johnson, L.; Martinson, M. Bacterial enrichments in surface films of freshwater lakes. J. Great Lakes Res. 1982, 8 (2), 323−325, DOI: 10.1016/S0380-1330(82)71973-2.

(26) Obernosterer, I.; Catala, P.; Reinthaler, T.; Herndl, G. J.; Lebaron, P. Enhanced heterotrophic activity in the surface microlayer of the Mediterranean Sea. Aquat. Microb. Ecol. 2005, 39 (3), 293−302, DOI: 10.3354/ame039293.

(27) Joux, F.; Agogué, H.; Obernosterer, I.; Dupuy, C.; Reinthaler, T.; Herndl, G.; Lebaron, P. Microbial community structure in the sea surface microlayer at two contrasting coastal sites in the northwestern Mediterranean Sea. Aquat. Microb. Ecol. 2006, 42 (1), 91−104, DOI: 10.3354/ame042091.

(28) Chin, W. C.; Orellana, M. V.; Verdugo, P. Spontaneous assembly of marine dissolved organic matter into polymer gels. Nature 1998, 391 (6667), 568−572, DOI: 10.1038/35345.

(29) Zimmermann-Timm, H. Characteristics, dynamics and importance of aggregates in rivers - an invited review. Int. Rev. Hydrobiol. 2002, 87 (2−3), 197−240, DOI: 10.1002/1522-2632(200205)87:2/33.0.CO;2-7.

(30) Cunliffe, M.; Murrell, J. C. The sea-surface microlayer is a gelatinous biofilm. ISME J. 2009, 3 (9), 1001−1003, DOI: 10.1038/ismej.2009.69.

(31) Elzerman, A. W.; Armstrong, D. E. Enrichment of Zn, Cd, Pb, and Cu in the surface microlayer of Lakes Michigan, Ontario, and Mendota. Limnol. Oceanogr. 1979, 24 (1), 133−144, DOI: 10.4319/lo.1979.24.1.0133.

(32) Wurl, O.; Obbard, J. P. A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. Mar. Pollut. Bull. 2004, 48 (11−12), 1016−1030, DOI: 10.1016/j.marpolbul.2004.03.016.

(33) Franklin, M. P.; McDonald, I. R.; Bourne, D. G.; Owens, N. J.; Upstill-Goddard, R. C.; Murrell, J. C. Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. Environ. Microbiol. 2005, 7 (5), 723−736, DOI: 10.1111/j.1462-2920.2004.00736.x.

(34) Cunliffe, M.; Schäfer, H.; Harrison, E.; Cleave, S.; Upstill-Goddard, R.; Murrell, J. C. Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary. ISME J. 2008, 2 (7), 776−789, DOI: 10.1038/ismej.2008.28.

(35) Obernosterer, I.; Catala, P.; Lami, R.; Caparros, J.; Ras, J.; Bricaud, A.; Dupuy, C.; van Wambeke, F.; Lebaron, P. Biochemical characteristics and bacterial community structure of the sea surface microlayer in the South Pacific Ocean. Biogeosciences 2008, 5 (3), 693−705, DOI: 10.5194/bg-5-693-2008.

(36) Hervas, A.; Casamayor, E. O. High similarity between bacterioneuston and airborne bacterial community compositions in a high mountain lake area. FEMS Microbiol. Ecol. 2009, 67 (2), 219− 228, DOI: 10.1111/j.1574-6941.2008.00617.x.

(37) Hörtnagl, P.; Pérez, M. T.; Sommaruga, R. Living at the border: A community and single-cell assessment of lake bacterioneuston activity. Limnol. Oceanogr. 2010, 55 (3), 1134−1144, DOI: 10.4319/lo.2010.55.3.1134.

(38) Hörtnagl, P.; Pérez, M. T.; Zeder, M.; Sommaruga, R. The bacterial community composition of the surface microlayer in a high mountain lake. FEMS Microbiol. Ecol. 2010, 73 (3), 458−467, DOI: 10.1111/j.1574-6941.2010.00904.x.

(39) Stolle, C.; Nagel, K.; Labrenz, M.; Jürgens, K. Succession of the sea-surface microlayer in the coastal Baltic Sea under natural and experimentally induced low-wind conditions. Biogeosciences 2010, 7, 2975−2988, DOI: 10.5194/bg-7-2975-2010.

(40) Hermansson, M.; Jones, G. W.; Kjelleberg, S. Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. Appl. Environ. Microbiol. 1987, 53 (10), 2338−2342.

(41) Jones, G. W.; Baines, L.; Genthner, F. J. Heterotrophic bacteria of the freshwater neuston and their ability to act as plasmid recipients under nutrient deprived conditions. Microb. Ecol. 1991, 22 (1), 15−25, DOI: 10.1007/BF02540210.

(42) Azevedo, J. S.; Araújo, S.; Oliveira, C. S.; Correia, A.; Henriques, I. Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine environment. Microb. Drug Resist. 2013, 19 (1), 64−71, DOI: 10.1089/mdr.2012.0084.

(43) Muschenheim, D. K.; Kepkay, P. E.; Kranck, K. Microbial growth in turbulent suspension and its relation to marine aggregate formation. Neth. J. Sea Res. 1989, 23 (3), 283−292, DOI: 10.1016/0077-7579(89)90049-5.

(44) Rijnaarts, H. H.; Norde, W.; Bouwer, E. J.; Lyklema, J.; Zehnder, A. J. Bacterial adhesion under static and dynamic conditions. Appl. Environ. Microbiol. 1993, 59 (10), 3255−3265.

(45) MacIntyre, S.; Alldredge, A. L.; Gotschalk, C. C. Accumulation of marine snow at density discontinuities in the water column. Limnol. Oceanogr. 1995, 40 (3), 449−468, DOI: 10.4319/lo.1995.40.3.0449.

(46) Wotton, R. S.; Preston, T. M. Surface films: Areas of water bodies that are often overlooked. BioScience 2005, 55 (2), 137−145, DOI: 10.1641/0006-3568(2005)055[0137:sfaowb]2.0.co;2.

(47) Alldredge, A. L.; Granata, T. C.; Gotschalk, C. C.; Dickey, T. D. The physical strength of marine snow and its implications for particle disaggregation in the ocean. Limnol. Oceanogr. 1990, 35 (7), 1415− 1428, DOI: 10.4319/lo.1990.35.7.1415.

(48) Milligan, T. G.; Hill, P. S. A laboratory assessment of the relative importance of turbulence, particle composition, and concentration in limiting maximal floc size and settling behaviour. J. Sea Res. 1998, 39 (3−4), 227−241, DOI: 10.1016/S1385-1101(97)00062-2.

(49) Henk, M. C. Method for collecting air-water interface microbes suitable for subsequent microscopy and molecular analysis in both research and teaching laboratories. Appl. Environ. Microbiol. 2004, 70 (4), 2486−2493,

DOI: 10.1128/aem.70.4.2486-2493.2004.

(50) Declerck, P.; Behets, J.; van Hoef, V.; Ollevier, F. Detection of *Legionella* spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. Water Res. 2007, 41 (14), 3159−3167, DOI: 10.1016/j.watres.2007.04.011.

(51) Cunliffe, M.; Harrison, E.; Salter, M.; Schäfer, H.; Upstill-Goddard, R. C.; Murrell, J. C. Comparison and validation of sampling strategies for the molecular microbial ecological analysis of surface microlayers. Aquat. Microb. Ecol. 2009, 57 (1), 69−77, DOI: 10.3354/ame01330.

(52) Cunliffe, M.; Whiteley, A. S.; Newbold, L.; Oliver, A.; Schäfer, H.; Murrell, J. C. Comparison of bacterioneuston and bacterioplankton dynamics during a phytoplankton bloom in a fjord mesocosm. Appl. Environ. Microbiol. 2009, 75 (22), 7173−7181, DOI: 10.1128/AEM.01374-09.

(53) Kjelleberg, S.; Stenström, T. A.; Odham, G. Comparative study of different hydrophobic devices for sampling lipid surface films and adherent microorganisms. Mar. Biol. 1979, 53 (1), 21−25, DOI: 10.1007/BF00386525.

(54) Hardy, J. T.; Coley, J. A.; Antrim, L. D.; Kiesser, S. L. A hydrophobic large-volume sampler for collecting aquatic surface microlayers: characterization and comparison with the glass plate method. Can. J. Fish. Aquat. Sci. 1988, 45 (5), 822−826, DOI: 10.1139/f88-099.

(55) Falkowska, L. Sea surface microlayer: a field evaluation of Teflon plate, glass plate and screen sampling techniques. Part 2. Dissolved and suspended matter. Oceanologia 1999, 41, 223−240.

(56) Zwart, G.; Crump, B. C.; Kamst-van Agterveld, M. P.; Hagen, F.; Han, S. K. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat. Microb. Ecol. 2002, 28 (2), 141−155, DOI: 10.3354/ame028141.

(57) Zhang, H.; DiBaise, J. K.; Zuccolo, A.; Kudrna, D.; Braidotti, M.; Yu, Y.; Parameswaran, P.; Crowell, M. D.; Wing, R.; Rittmann, B. E.; Krajmalnik-Brown, R. Human gut microbiota in obesity and after gastric bypass. Proc. Natl. Acad. Sci. U. S. A. 2009, 106 (7), 2365−2370, DOI: 10.1073/pnas.0812600106.

(58) Bartram, A. K.; Lynch, M. D.; Stearns, J. C.; Moreno-Hagelsieb, G.; Neufeld, J. D. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. Appl. Environ. Microbiol. 2011, 77 (11), 3846−3852, DOI: 10.1128/AEM.02772-10.

(59) Cole, J. R.; Wang, Q.; Cardenas, E.; Fish, J.; Chai, B.; Farris, R. J.; Kulam-Syed-Mohideen, A. S.; McGarrell, D. M.; Marsh, T.; Garrity, G. M.; Tiedje, J. M. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 2009, 37 (Suppl 1), D141−145, DOI: 10.1093/nar/gkn879.

(60) Abramoff, M. D.; Magelhaes, P. J.; Ram, S. J. Image processing with ImageJ. Biophotonics Int. 2004, 11 (7), 36−42.

(61) Environment Canada. Analytical methods manual; Inland Waters Directorate, Water Quality Branch: Ottawa, 1979.

(62) Moore, T. R. The spectrophotometric determination of dissolved organic carbon in peat waters. Soil Sci. Soc. Am. J. 1985, 49 (6), 1590 − 1592,

DOI: 10.2136/sssaj1985.03615995004900060054x.

(63) Kepner, R. L.; Pratt, J. R. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol. Rev. 1994, 58 (4), 603−615.

(64) Callieri, C.; Stockner, J. G. Freshwater autotrophic picoplankton: a review. J. Limnol. 2002, 61 (1), 1−14, DOI: 10.4081/jlimnol.2002.1.

(65) Maki, J. S.; Remsen, C. C. A membrane adsorption-SEM technique for observing neuston organisms. Microb. Ecol. 1983, 9 (2), 177−183, DOI: 10.1007/BF02015130.

(66) Newton, R. J.; Jones, S. E.; Eiler, A.; McMahon, K. D.; Bertilsson, S. A guide to the natural history of freshwater lake bacteria. Microbiol. Mol. Biol. Rev. 2011, 75 (1), 14−49, DOI: 10.1128/MMBR.00028-10.

(67) Reche, I.; Ortega-Retuerta, E.; Romera, O.; Pulido-Villena, E.; Morales-Baquero, R.; Casamayor, E. O. Effect of Saharan dust inputs on bacterial activity and community composition in Mediterranean lakes and reservoirs. Limnol. Oceanogr. 2009, 54 (3), 869−879, DOI: 10.4319/lo.2009.54.3.0869.

(68) Peter, H.; Hörtnagl, P.; Reche, I.; Sommaruga, R. Bacterial diversity and composition during rain events with and without Saharan dust influence reaching a high mountain lake in the Alps. Environ. Microbiol. Rep. 2014, 6 (6), 618-624. DOI: 10.1111/1758-2229.12175.

(69) Voorhies, A. A.; Biddanda, B. A.; Kendall, S. T.; Jain, S.; Marcus, D. N.; Nold, S. C.; Sheldon, N. D.; Dick, G. J. Cyanobacterial life at low O_2: community genomics and

function reveal metabolic versatility and extremely low diversity in a Great Lakes sinkhole mat. Geobiology 2012, 10 (3), 250−267, DOI: 10.1111/j.1472-4669.2012.00322.x.

(70) Elasri, M. O.; Miller, R. V. Study of the response of a biofilm bacterial community to UV radiation. Appl. Environ. Microbiol. 1999, 65 (5), 2025−2031.

(71) Harrison, J. J.; Ceri, H.; Turner, R. J. Multimetal resistance and tolerance in microbial biofilms. Nat. Rev. Microbiol. 2007, 5 (12), 928−938, DOI: 10.1038/nrmicro1774.

(72) Haack, E.; Warren, L. A. Biofilm hydrous manganese oxyhydroxides and metal dynamics in acid rock drainage. Environ. Sci. Technol. 2003, 37 (18), 4138−4147, DOI: 10.1021/es026274z.

(73) Stewart, P. S.; Franklin, M. J. Physiological heterogeneity in biofilms. Nat. Rev. Microbiol. 2008, 6 (3), 199−210, DOI: 10.1038/ nrmicro1838.

(74) Ma, S.; Banfield, J. F. Micron-scale Fe^{2+}/Fe^{3+}, intermediate sulfur species and O_2 gradients across the biofilm−solution−sediment interface control biofilm organization. Geochim. Cosmochim. Acta 2011, 75 (12), 3568−3580, DOI: 10.1016/j.gca.2011.03.035.

(75) Wheeler, J. R. Formation and collapse of surface films. Limnol. Oceanogr. 1975, 20 (3), 338−342, DOI: 10.4319/lo.1975.20.3.0338.

(76) Tang, K. W.; Dziallas, C.; Grossart, H. P. Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment. Environ. Microbiol. 2011, 13 (2), 378−390, DOI: 10.1111/j.1462-2920.2010.02335.x.

(77) Paerl, H. W.; Pinckney, J. L. A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. Microb. Ecol. 1996, 31 (3), 225−247, DOI: 10.1007/BF00171569.

(78) Balzano, S.; Statham, P. J.; Pancost, R. D.; Lloyd, J. R. Role of microbial populations in the release of reduced iron to the water column from marine aggregates. Aquat. Microb. Ecol. 2009, 54 (3), 291−303, DOI: 10.3354/ame01278.

(79) Elliott, A. V. C.; Plach, J. M.; Droppo, I. G.; Warren, L. A. Collaborative microbial Fe-redox cycling by pelagic floc bacteria across wide ranging oxygenated aquatic systems. Chem. Geol. 2014, 366, 90−102, DOI: 10.1016/j.chemgeo.2013.11.017.

(80) Luettich, R. A.; Harleman, D. R. F.; Somlyody, L. Dynamic behavior of suspended sediment concentrations in a shallow lake perturbed by episodic wind events. Limnol. Oceanogr. 1990, 35 (5), 1050−1067, DOI: 10.4319/lo.1990.35.5.1050.

(81) Birch, G.; O'Hea, L. The chemistry of suspended particulate material in a highly contaminated embayment of Port Jackson (Australia) under quiescent, high-wind and heavy-rainfall conditions. Environ. Geol. 2007, 53 (3), 501−516, DOI: 10.1007/s00254-007-0662-5.

CHAPTER 4: WIDESPREAD SOLUBLE AND SOLID FE(III)-REDUCING ACTIVITY OF BACTERIA IN OXIC LITTORAL REGIONS OF FRESHWATER LAKES

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4.1 Abstract

Bacteria capable of reducing soluble Fe(III) were enriched from the surface microlayer (SML; neuston layer) and proximate underlying waters (0.5 m depth) in oxic littoral regions of two physically and geochemically contrasting freshwater lakes, Coldspring Lake (nature reserve) and Lake Ontario (urban setting) over two consecutive years. Enrichment communities comprised a diverse group of genera belonging to the phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, most of which are common in freshwater environments and include metabolically flexible facultative anaerobes. Wastewater- and sediment-associated putative Fe(III) reducers (e.g. *Cloacibacterium* and *Clostridium*, respectively) were enriched only from the more impacted, shallower, and higher energy Lake Ontario littoral site. Notably absent from all enrichment communities were well-studied, classic sedimentary Fe(III)-reducing bacteria, i.e. *Geobacter* spp. and *Shewanella* spp.. The capacity for metabolically more challenging solid Fe(III) reduction appeared to be controlled by the availability of soluble Fe(III). No solid Fe(III)-reducing bacteria were enriched from the SML of Coldspring Lake, where dissolved (<0.2 μm) Fe(III) concentrations (1.9 ± 0.4 μM) were significantly ($P < 0.05$) higher than those observed at 0.5 m depth (0.9 ± 0.3 μM), as well as in the SML and at 0.5 m depth at Lake Ontario (0.5 ± 0.2 μM and 0.4 ± 0.2 μM,

respectively), from which solid Fe(III)-reducing bacteria were enriched. Imaging identified the occurrence of large (>500 μm^2) flocs, the abundance of which directly corresponded to the magnitude of solid Fe(III) reduction by enrichment communities between depths in each lake. These results support a physical role for floc in providing suitable microenvironments for O_2-sensitive microbial Fe(III) reduction under bulk oxic conditions and highlight the much wider environmental occurrence of microbial Fe reduction than previously thought.

4.2 Introduction

Microbial Fe(III) reduction in oxic circumneutral surface waters has traditionally not been considered to play a significant role in Fe transformations because many well-studied Fe(III)-reducing bacteria are obligate or facultative anaerobes originally recovered from anoxic environments (Lovley 2013). However, it is now emerging that Fe(III)-reducing bacteria may contribute substantially to Fe transformation in oxic aquatic settings by operating within suspended flocs (Balzano et al. 2009; Lu et al. 2013; Elliott and Warren 2014; Elliott et al. 2014; Ellwood et al. 2014). Flocs are mixed inorganic-organic aggregates dominated by an intrinsic active microbial community and associated organic products (Liss et al. 1996; Droppo 2001). They are ubiquitous in aquatic systems and are hotspots of biogeochemical activity relative to their surrounding milieu (Simon et al. 2002). Within flocs, micrometer-scale gradients in O_2 concentration can develop where the local consumption of O_2 by respiring heterotrophic biota exceeds its replenishment via transport from surrounding oxic waters (Paerl and Pinckney 1996; Simon et al. 2002). The recovery of active bacteria capable of metabolisms inhibited by O_2 and detection of reduced Fe and S species in association with flocs in oxic circumneutral aquatic environments (Shanks and Reeder 1993;

Balzano et al. 2009) indicate that floc-based O_2-depleted microzones capable of supporting microbial Fe(III) reduction commonly occur in oxic settings.

Within well-oxygenated surface waters, flocs are known to accumulate in the surface microlayer (SML), or neuston layer, a biogeochemically distinctive millimeter-thick microbial habitat situated directly beneath the air-water interface (Drudge and Warren, 2014). Relative to proximate underlying oxic waters (i.e. <0.5 m depth), the SML is regularly enriched with organic and inorganic nutrients and contains a highly metabolically active microbial community (Södergren 1993; Münster et al. 1997; Reinthaler et al. 2008; Baastrup-Spohr and Staehr 2009) that forms biofilm structures from which flocs are generated (Cunliffe and Murrell 2009; Drudge and Warren 2014). Although its SML enrichment remains poorly characterized, Fe is a component of terrigenous mineral dusts and industrial aerosol emissions and so is constantly deposited to and thus concentrated in the microlayer of surface waters (Mahowald et al. 2009; Boyd et al. 2010). Dust deposited to freshwater lakes can be enriched in Fe relative to their underlying bedrock, indicating that atmospheric deposits can serve as a Fe source (Ballantyne et al. 2011). Additionally, as Fe is known to closely associate with microbial cells and their organic products in flocs (Plach et al. 2011; Elliott et al. 2012) and other aquatic biofilm environments (Dong et al. 2000; Chan et al. 2009), it is expected to accumulate along with them in the SML. Thus, the SML should be able to support an abundant heterotrophic microbial community capable of high rates of O_2 respiration necessary to generate floc-based O_2-depleted microenvironments suitable for microbial Fe(III) reduction, while accumulating the substrates (Fe, nutrients) necessary to support Fe(III) reduction in microlayer flocs. As the SML regularly accumulates inorganic and organic contaminants (Armstrong and Elzerman 1982; Wurl and Obbard 2004; Cuong et

al. 2008; Ju et al. 2008), it is a key location for the control of contaminant behaviour in aquatic systems, including their exchange with the atmosphere. If microbial Fe(III) reduction does occur within the SML, it would be an important process affecting biogeochemical cycling of nutrients and contaminants, impacting water column characteristics as well as lake-atmosphere interactions. Further, the much different bulk O_2 status and concentrated labile organic carbon content of the SML and potential for distinct floc-based microbial Fe cycling (Elliott et al. 2014) relative to anoxic sedimentary environments where microbial Fe(III) reduction is classically considered, suggest that both soluble Fe(III) (i.e. sorbed to organic matter and/or minerals) as well as amorphous Fe(III) oxyhydroxide minerals (i.e. highly reactive biogenic Fe oxides) (Ferris 2005; Gault et al. 2011) occur within the SML. Thus the SML may support different Fe(III)-reducing bacteria than those dominantly found in anoxic environments, i.e. *Geobacter* spp. (Lovley and Phillips 1988; Coates et al. 1996; Nevin et al. 2005; Prakash et al. 2010) or *Shewanella* spp. (Myers and Nealson 1988; Venkateswaran et al. 1998; Kim et al. 2012). Recent examination of pelagic flocs from several different freshwater systems identified the presence of a wide variety of Fe(III)-reducing bacteria that did not include *Geobacter* or *Shewanella* spp. (Elliott et al. 2014), identifying differentiated ecology of Fe(III) reducers in pelagic versus anoxic sedimentary contexts.

The objectives of this combined field and laboratory investigation were to (1) investigate whether Fe(III)-reducing bacteria occur in the SML and proximate underlying oxic waters (0.5 m depth) of freshwater lakes through functional enrichment, (2) identify, if present, differences in the composition of SML Fe(III)-reducing bacterial enrichments and their capacity for soluble and solid phase Fe(III) reduction between depths and systems, and (3) assess how system-specific physical and/or geochemical characteristics influence the

composition and capacity of SML Fe(III) reducers collected from the littoral zone of two physically and geochemically contrasting freshwater systems, an urban beach (Sunnyside Beach) on Lake Ontario and a small sheltered lake (Coldspring Lake) in a remote nature reserve.

4.3 Materials and Methods

4.3.1 Site Descriptions

Coldspring Lake is a small (<1 km^2), shallow (average depth ~3 m, maximum depth ~7 m), and organic- and Fe-rich (4.2-4.5 mg/L dissolved organic carbon (DOC; <0.45 μm), 3.0-3.6 μM Fe) wetland-associated Canadian Shield lake (pH ~7.0) sheltered by bordering dense forest and located in a remote northwestern region (45°51'12"N 78°49'24"W) of Algonquin Provincial Park (Ontario) designated as a nature reserve zone (Drudge and Warren 2014). In contrast, Sunnyside Beach is a relatively organic- and Fe-poor (2.5-3.2 mg/L DOC, 1.2-1.6 μM Fe) manufactured public sand beach on the northwestern shore of Lake Ontario (~19,000 km^2, pH ~8.4, 43°38'14"N 79°27'21"W) in downtown Toronto (population ~3 million), impacted by wastewater effluents and traffic- and industry-produced air pollution (Drudge and Warren 2014). The fetch at Sunnyside Beach was >50 km based on the south/west wind direction at time of sampling, compared to <1 km at Coldspring Lake. Differences between the two sites with respect to their physical and geochemical characteristics should influence the composition and capacity of their Fe(III)-reducing bacterial communities. A previous investigation of these sites found that whole-day concentrations of DOC were higher in the SML compared to 0.5 m depth at Sunnyside

Beach but lower in the SML compared to 0.5 m depth at Coldspring Lake (Drudge and Warren 2014).

4.3.2 Sample Collection

Water samples were collected to (1) enrich for and subsequently characterize soluble and solid Fe(III)-reducing bacteria and (2) characterize water column concentrations of Fe and PO_4^{3-}, as well as floc abundance. Samples were collected from the SML and underlying water column (0.5 m depth) at littoral sites on both lakes, ~5 m from the shore at Sunnyside Beach and ~15 m from the shore at Coldspring Lake (water column depth was ~3 m at Coldspring Lake and ~1 m at Sunnyside Beach, >1% of PAR irradiance reached the sediment-water interface at both sites). Sampling was carried out in the morning under relatively quiescent conditions in consecutive summers (2010 and 2011) during a single excursion. Coldspring Lake was sampled on August 12 at 7:00 in 2010, as well as on July 21 at 6:00 in 2011. Sunnyside Beach was sampled at 10:00 on July 21, 2010 and August 10, 2011. Water column dissolved O_2 concentrations were characterized *in situ* using a YSI 6600 V2-2 Multiparameter Water Quality Sonde (YSI Incorporated). At both sites, the water column was not stratified and the sampling depths were well oxygenated, with dissolved O_2 saturation consistently measured to be >105% at Sunnyside Beach and between 95-100% at Coldspring Lake during all sampling periods.

Bacteria capable of reducing soluble Fe(III) were enriched from SML and 0.5 m depth water samples using a Fe(III) citrate-based M1 medium that contained 10 mM Na acetate as a carbon substrate, 25 mM Fe (III) citrate as electron acceptor, and 0.01% (w/v) yeast extract to stimulate heterotrophic growth (Kostka and Nealson 1998). Membrane

sampling of the SML bacterial community was carried out according to Cunliffe et al. (2011) using nucleation-track polycarbonate membrane filters (47 mm diameter, 0.1 μm pore size; Isopore; Millipore). This method achieved a SML sampling depth of ~40 μm (Crawford et al. 1982; Franklin et al. 2005; Cunliffe et al. 2009). During each sampling period, the SML was randomly sampled using five filters within a ~1 m^2 area, which were then used to inoculate a sterile 15 mL Falcon tube (BD Biosciences) containing 13 mL of soluble Fe(III) citrate-based enrichment medium. To sample the bacterial community at 0.5 m depth, water samples were collected using a sterilized Van Dorn sampler. A sterile 15 mL Falcon tube containing 13 mL of soluble Fe(III) citrate-based enrichment medium was inoculated with a 350 uL aliquot of the 0.5 m depth water sample, which was approximately the same volume collected from the SML for the same analysis. Following their inoculation with water samples, as described previously, enrichment tubes were incubated at room temperature in the dark to simulate the substantial attenuation of incident sunlight within larger flocs where microbial Fe(III) reduction would be expected to be active in oxic waters (Kühl et al. 1996; Loge et al. 1999). This approach also minimized the complicating effects of photochemical Fe transformations and photosynthetic O_2 production on the accurate measurement of microbial Fe(II) production in microcosms. Tubes were tightly capped and left stationary, resulting in the development of microoxic (<15% O_2 saturation) conditions within 48 h, as measured using the dissolved O_2 sensor on a YSI 6600 V2-2 Multiparameter Water Quality Sonde (YSI Incorporated). Soluble Fe(III) reduction was visually evidenced by the clearing of orange-brown soluble Fe(III) citrate from solution and formation of a brown precipitate over the course of a 720 h incubation, with these changes absent in abiotic controls (Figure S.4.1). Enrichments were transferred (10% of total volume as an inoculum) into tubes of newly

prepared enrichment medium every month. At least six successive transfers were carried out prior to enrichment characterization.

Bulk water samples (~2.5-3 L) for the measurement of Fe and PO_4^{3-} concentrations and floc abundance (n=2 sampling periods at each site over two years) were collected from the SML using a sheet surface sampler (Drudge and Warren 2014). Concurrent with SML sampling, water samples (~2.5-3 L) were collected from 0.5 m depth using a Van Dorn sampler. For each sampling period, bulk water samples from the SML and from 0.5 m depth were collected into 4 L low-density polyethylene carboys (Reliance Products) and subsequently aliquoted for Fe, PO_4^{3-}, and floc imaging (abundance) analyses. To minimize nutrient and Fe contamination, all equipment and containers used for the collection and storage of water samples were soaked in 4% (v/v) HCl for >24 h and then rinsed eight times with ultrapure water (18.2 MΩ cm; Milli-Q; Millipore) before use. Operational field blanks of ultrapure water were also assessed and indicated negligible contamination for each geochemical analyte. Triplicate aliquots for dissolved (<0.2 μm) Fe analysis (n=2 sampling periods at each site over two years) were transferred from carboys into acid-washed syringes, sequentially filtered through 0.45- and 0.2-μm syringe filters (Millipore) into 15 mL polypropylene Falcon tubes (BD Biosciences), and acidified to 2% HNO_3 (v/v) using trace element grade concentrated (67%) nitric acid (Fisher Scientific). Triplicate aliquots for total Fe analysis (n=2 sampling periods at each site over two years) were directly transferred from carboys into 15 mL Falcon tubes and acidified. Triplicate aliquots (n=2 sampling periods at each site in 2010 only) for the measurement of floc abundance were transferred from carboys into 50 mL polypropylene Falcon tubes. The remaining water samples in carboys

were retained for PO_4^{3-} analysis. Aliquots were frozen at -20°C in the dark until analysis, while carboys were stored at 4°C in the dark until analysis.

4.3.3 Floc Analyses

To quantify the abundance and size distribution of suspended flocs collected in the field from the SML and at 0.5 m depth in 2010, water samples of known volume were passed through polycarbonate membrane filters (0.1 μm pore size) by vacuum filtration and imaged using a Leica DMRA microscope (Leica Microsystems). Bright field images were captured using OpenLab software (version 5.1) (PerkinElmer). Images of at least 35 fields of view were captured at 1000× magnification for Sunnyside Beach samples and at 100× magnification for Coldspring Lake samples. Floc abundance and size (i.e. area based on a two-dimensional top-down view) were measured using ImageJ software (version 1.43) (Abramoff et al. 2004; http://rsb.info.nih.gov/ij/).

4.3.4 Aqueous Fe and PO_4^{3-} Analyses

Fe (Fe(II) + Fe(III)) concentrations (total and dissolved (<0.2 μm)) for both depths in each lake were measured in triplicate, previously acidified water samples using the colorimetric FerroVer method based on 1,10-phenanthroline complexation (Hach Company). Total soluble reactive phosphate (PO_4^{3-}) was measured in triplicate using a spectrophotometric Hach method (PhosVer 3 ascorbic acid method) as per the manufacturer's instructions. Where reported, Fe and PO_4^{3-} concentration values are given as mean ± standard error.

4.3.5 Solid Fe(III) Microcosm Experiments

The ability of soluble Fe(III)-reducing enrichment communities to also reduce solid Fe(III), as well as identification of their bacterial community composition and physical structure (i.e. biofilm formation), were examined in experimental microcosms set up in triplicate for enrichments from each lake/depth/year (n=8 enrichment communities), along with an abiotic control, using a growth medium based on a synthetic mixture of amorphous/crystalline Fe oxyhydroxide (FeOOH) minerals. This mixture, intended to mimic forms of Fe found in circumneutral, non-sulfidic, freshwater environments such as those sampled in this study, was prepared according to a method by Schwertmann and Cornell (2000). Briefly, a 0.2 M solution of ferric chloride was slowly neutralized with a 1 M NaOH solution to a pH of ~8. The resulting precipitate was washed six times with ultrapure water (18.2 MΩ cm; Milli-Q; Millipore) to remove chloride ions, and stored at 4°C in the dark until use. A medium consisting of 0.1% w/v tryptone, 0.02% w/v yeast extract, 0.3% w/v sodium acetate, and 15% v/v settled FeOOH mixture slurry (~50 mM Fe(III)) was prepared using tap water and sterilized by autoclaving prior to use. X-ray powder diffraction analysis revealed that autoclaved medium mineral solids consisted of >75% amorphous Fe, ~10% ferrihydrite/feroxyhite, ~5% goethite, and ~10% other phases (each of which individually constituted <2 %).

Triplicate 15 mL Falcon tubes, filled with 13 mL of the FeOOH mixture medium, were inoculated with 100 μL (1% v/v inoculum) of previously established soluble Fe(III) citrate-reducing enrichments. Uninoculated tubes were used as a sterile control. Following their set up, tubes were tightly capped, left stationary, and incubated over 720 h at room temperature in the dark to achieve microoxic (<15% O_2 saturation) conditions. Reduction of

FeOOH solids was visually indicated by the appearance of an orange-brown tinge in the aqueous solution above settled FeOOH extensively colonized by enrichment bacteria, which was absent from abiotic controls (Figure S.4.1). Further, FeOOH reduction was quantitatively assessed by the detection of aqueous Fe(II), using the ferrozine method described by Viollier et al. (2000), at concentrations significantly ($P < 0.05$) higher than abiotic controls during the incubation period.

Mean concentrations of aqueous Fe(II) (n=3 per enrichment) were measured in microcosms at 72 to 120 h intervals. Fe(II) concentrations were measured (Viollier et al. 2000) following microcosm agitation (inverting microcosm tubes several times), in order to detect Fe(II) in aqueous solution as well as weakly associated with enrichment communities (García-Balboa et al. 2011). The pH of all microcosms inoculated with Fe(III)-reducing enrichments did not differ by more than ± 0.5 over the course of the 720 h incubation period, with the pH of all microcosms decreasing from ~8.5 to 7, indicating that the effects of microbial Fe transformations on pH and the effects of pH on Fe(II) partitioning between solid phases and aqueous solution did not differ appreciably between enrichment microcosms. To minimize contamination, all equipment and containers used for the measurement of Fe(II) concentrations were soaked in 4% (v/v) HCl for >24 h and then rinsed eight times with ultrapure water (18.2 MΩ cm; Milli-Q; Millipore) before use. Concurrent with the measurement of Fe(II) production, bulk enrichment community growth was estimated by diluting 15 uL aliquots from agitated microcosms in 135 uL (1:10 dilution) of nonselective YPG growth media (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) that included 0.01% w/v iodonitrotetrazolium chloride (INT) (Sigma-Aldrich) as a colorimetric indicator of bacterial cell abundance (Hatzinger et al. 2003). Following a 48 h

incubation period at room temperature, these preparations were diluted 1:10 in ultrapure water and the absorbance of the resulting solution was measured at 480 nm.

To confirm the growth and viability of the microcosm-based enrichment communities and characterize their physical structure, intact (viable) and membrane-compromised microbial cells sampled at the end of the microcosm incubation periods (t=720 h) were imaged using the LIVE/DEAD BacLight nucleic acid staining technique according to the manufacturer's instructions (Molecular Probes, Inc.). Briefly, aliquots from agitated microcosms were diluted 1:10 in sterile water to a final volume of 1 mL, to which 0.5 μL of each staining solution was added. Samples were incubated for 15 minutes prior to mounting and imaging. Slides were viewed using a Leica DMRA microscope equipped with GFP and TX2 filter cubes (470/40 nm excitation and 525/50 nm barrier, and 560/40 nm excitation and 645/75 nm barrier, respectively).

The composition of microcosm bacterial communities was then determined by 16S rRNA gene sequencing. For each enrichment, total community DNA was isolated from 450 μL of agitated microcosms (150 μL aliquots from each of the three replicate microcosms) using the PowerSoil DNA Isolation Kit (MO Bio Laboratories) according to the manufacturer's instructions. Bacterial 16S rRNA genes were selectively amplified from total community DNA by PCR using the 27F/1492R primer set according to Zwart et al. (2002), and then cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). At least 40 clones containing inserts were sequenced (>900 nucleotides) for each sample (MOBIX Lab, McMaster University, Hamilton, Ontario, Canada). These sequences were published in the GenBank database under the accession numbers KM091641 to KM091668. The closest database matches to each sequence were obtained using BLAST

(http://blast.ncbi.nlm.nih.gov/). As universal functional gene markers for Fe(III) reduction are not known, putative Fe(III)-reducing members of microcosm bacterial communities were identified based on their 16S rRNA gene sequence similarity to known Fe(III) reducers. Alignment of sequences (~900 base positions) was performed using ClustalX2 (Larkin et al. 2007). Following alignment, phylogenetic trees were generated by maximum likelihood analysis (n=1000 bootstraps) based on the Tamura-Nei substitution model using MEGA version 6 (Tamura et al. 2013).

4.3.6 Statistical Analysis

Statistical analysis was carried out using StatPlus:mac LE (AnalystSoft, http://www.analystsoft.com/). Statistical comparisons between lakes and between water column depths were each made by one-way ANOVA, followed by Tukey's post hoc test.

4.4 Results and Discussion

In consecutive years (2010 and 2011), bacteria capable of reducing soluble Fe(III) under microoxic (<15% O_2 saturation) conditions were successfully enriched from both the SML and proximate underlying oxic water column (0.5 m depth) from Sunnyside Beach, Lake Ontario (>105% O_2) and Coldspring Lake (95-100% O_2), establishing the potential for bacterial Fe(III) reduction to occur in the upper water column and specifically at the air-water interface of aquatic systems. As the SML is a collection site for nutrients, contaminants and Fe, and a gatekeeper for atmosphere-hydrosphere exchange, the presence of Fe(III)-reducing bacteria in the microlayer is indicative of the importance of this interfacial habitat for biogeochemical cycling related to Fe. Further, the enrichment of SML Fe(III)-reducing

bacteria under microoxic conditions indicates that the microlayer can harbor anaerobic microorganisms (e.g. in O_2-depleted microenvironments within flocs), expanding the potential metabolic activities carried out by bacteria at the air-water interface to include those inhibited by O_2 (e.g. the use of non-O_2 terminal electron acceptors).

Soluble Fe(III)-reducing enrichment communities from both lakes, depths, and years included a phylogenetically diverse group of bacteria affiliated with the alpha, beta, and gamma classes of the phylum *Proteobacteria*, as well as the phyla *Firmicutes* and *Bacteroidetes* (Figure 4.1), indicating a widespread capacity for Fe(III) reduction among bacteria inhabiting the upper water column of freshwater lakes. Most of the dominant (i.e. constituting >25% of clone library sequences) enrichment community members belonged to genera that are commonly found in aquatic environments and include metabolically flexible facultative anaerobes capable of fermentation or respiration using various terminal electron acceptors in addition to O_2 (e.g. *Achromobacter*, *Aeromonas*, *Cloacibacterium*, *Comamonas*, *Pseudomonas*, *Serratia*) (Figure 4.1). A capacity for both aerobic and anaerobic growth would permit these littoral Fe(III)-reducing bacteria to inhabit and move between floc-based low O_2 microenvironments and well-oxygenated ambient waters, persisting throughout the dynamic generation and breakdown of flocs that takes place in the high energy, wind- and sunlight-exposed upper water column of lakes (Drudge and Warren 2014). Additionally, in agreement with other studies of the water column of freshwater lakes (Lehours et al. 2009; Elliott et al. 2014) well-studied dissimilatory Fe(III)-reducing bacteria associated with anoxic sediments (e.g. *Geobacter* spp., *Shewanella* spp., Fe- and S-reducing *Deltaproteobacteria*) were not detected. The absence of these taxa may reflect their greater oxygen intolerance as well as the predominance of complex carbon substrates in the oxic upper water column of lakes (e.g.

microbially-derived floc components) relative to anoxic sedimentary environments. The presence of the obligate Fe(III)-reducing genus *Geobacter* in Fe(III)-reducing enrichments has been linked to the use of relatively simple carbon sources, while putative Fe(III)-reducing *Enterobacteriaceae* and *Firmicutes*, present in enrichments in this study (Figure 4.1), were major constituents of enrichments containing more complex glucose or mixed carbon sources (Lentini et al. 2012). Collectively, these findings are consistent with the presence of a distinct phylogenetically diverse and metabolically versatile group of facultative Fe(III)-reducing bacteria in the bulk oxic upper water column of littoral freshwater sites adapted to both planktonic (high O_2) and floc-based (low O_2) growth and the utilization of complex organic substrates, which distinguishes them from populations typically found in the underlying anoxic bed sediments of freshwater lakes.

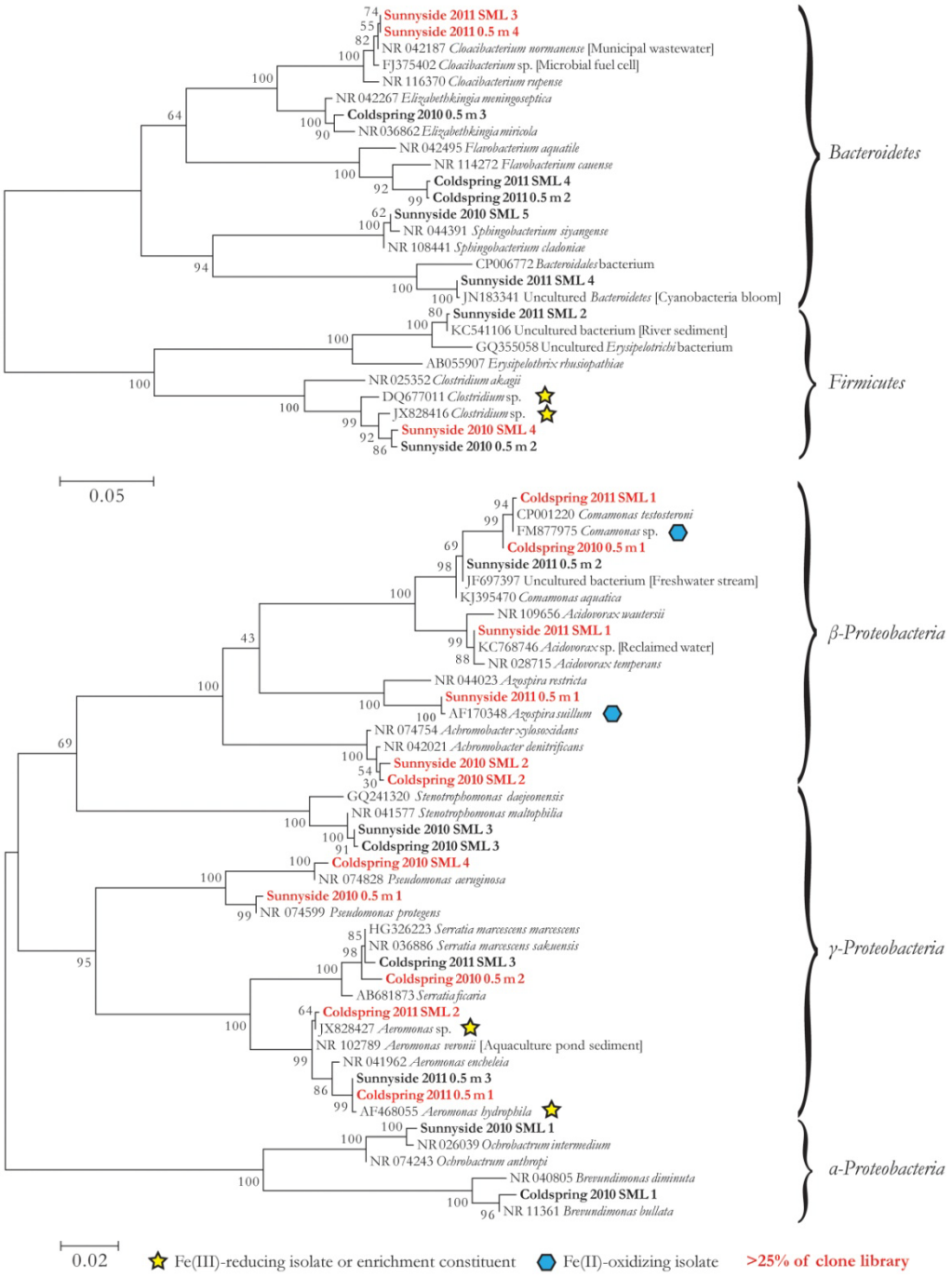

Figure 4.1 Placement of bacterial 16S rRNA gene sequences from solid Fe(III)-containing microcosms inoculated with SML and 0.5 m depth soluble Fe(III)-reducing enrichment communities (n=3) (t=end) with other known bacteria based on the maximum likelihood method of analysis. Bootstrap values (percentage) for nodes are based on 1000 resamplings. Scale bars represent fixed mutations per nucleotide position.

Interestingly, the composition of enriched soluble Fe(III)-reducing communities consistently differed between the SML and 0.5 m depth in both systems. No overlap occurred between enrichment bacterial 16S rRNA gene clone libraries from the 0.5 m depth and the SML for either system in 2010, and only a single sequence match was shared between libraries from each depth for either system in 2011 (Figure 4.1). Further, there was also minimal overlap of enrichment communities between the two lakes at both depths. Coldspring Lake SML and 0.5 m depth enrichment clone libraries consisted predominantly of bacterial genera commonly found within the water column of freshwater lakes (e.g. *Achromobacter*, *Aeromonas*, *Comamonas*, and *Pseudomonas*), while sediment- and wastewater-associated bacteria (e.g. *Cloacibacterium* and *Clostridium*) dominated Sunnyside Beach SML and 0.5 m depth libraries (Figure 4.1). Unlike soluble Fe(III)-reducing enrichment communities sampled from both depths and years at Coldspring Lake, those from Sunnyside Beach at both depths in 2010 included a member of the anaerobic genus *Clostridium* (Figure 4.1). This *Clostridium* sp. matched (98% sequence identity) a constituent of a Fe(III)-reducing enrichment recovered from freshwater pond sediment (Lentini et al. 2012), indicating that it was likely principally responsible for Fe(III) reduction by Sunnyside Beach 2010 SML and 0.5 m depth enrichment communities. The genus *Clostridium* and other bacteria belonging to the phylum *Firmicutes* are typically a minor component of bacterial communities in the upper water column of freshwater lakes, being more commonly found in freshwater bed sediments (Newton et al. 2011). The littoral sampling location at Sunnyside Beach was very shallow (~1 m depth) and less sheltered from wind-driven mixing compared to the Coldspring Lake sampling location (~3 m depth) (Drudge and Warren 2014). Thus, the resuspension of bed sediments at Sunnyside Beach may have resulted in appreciable transport of sedimentary,

floc-based Fe(III)-reducing *Firmicutes* (e.g. *Clostridium*) to the overlying shallow water column at this site. Consistent with this notion, bed sediment agitation and resuspension in shallow aquatic systems (~1 m depth) has been demonstrated to transport substantial quantities of floc and large numbers of sediment-associated bacteria into the overlying water column (Arfi and Bouvy 1995; Crabill et al. 1999; Whitman et al. 2006; Plach et al. 2011). Additionally, unlike Coldspring Lake enrichment communities from both depths and years, the majority of sequences in the Sunnyside Beach 2011 SML and 0.5 m depth enrichment clone libraries matched either an *Acidovorax* sp. isolated from reclaimed water (99% sequence identity) or both the type strain of *Cloacibacterium normanense*, isolated from wastewater (Allen et al. 2006), and a *Cloacibacterium* sp. found in a microbial fuel cell (99% sequence identity for both) (Figure 4.1). As Fe(III)-reducing bacteria are known to participate in microbial fuel cells (Logan 2009), the previous detection of *C. normanense* in this setting suggests that is capable of transferring electrons to extracellular acceptors such as Fe(III) and thus principally responsible for Fe(III) reduction by Sunnyside Beach 2011 SML and 0.5 m depth enrichment communities. The allochthonous origins of these bacteria and their apparent dominant presence in Fe(III)-reducing enrichment communities indicate that wastewater was the source of Fe(III)-reducing bacteria at the Sunnyside Beach site. Wastewater occasionally contaminates urban Sunnyside Beach via neighbouring combined sewer overflows, but does not appreciably impact remote Coldspring Lake, implicating anthropogenic contamination as a physical system characteristic that influences the identities of Fe(III)-reducing bacteria in the littoral zone of freshwater lakes. Wastewater from combined sewer overflows is typically rich in flocs (Michelbach 1995; Droppo et al. 2002), which may serve as a vehicle for the introduction of Fe(III)-reducing bacteria to littoral freshwater settings. Thus the differing

composition of enriched Fe(III)-reducing bacteria from each lake can be explained in part by the greater impact of wastewater contamination and bed sediment resuspension at Sunnyside Beach relative to Coldspring Lake.

While enrichment communities from both depths within each lake in consecutive years were capable of reducing soluble Fe(III), these communities were not similar across the two lakes with respect to their ability to reduce solid Fe(III). Although Sunnyside Beach SML enrichment communities from both years were capable of reducing a mixture of FeOOH solids, evidenced by higher Fe(II) concentrations ($P < 0.05$) in enrichment microcosms compared to sterile controls, the same capacity for solid Fe(III) reduction was absent from Coldspring Lake SML enrichments from both years (Table 4.1). This indicates that the functional capabilities of these two SML communities to access solid Fe(III) sources were very different, despite their shared ability to reduce soluble Fe(III). Further emphasizing the distinctiveness of the non-solid Fe(III)-reducing Coldspring Lake SML enrichment community, 0.5 m depth enrichment communities from both lakes were capable of reducing solid Fe(III) (Table 4.1). These results establish the widespread capacity for soluble and solid Fe(III) reduction by littoral bacteria. However, they also indicate a nuanced capacity for solid Fe(III) reduction that is neither water column zone (i.e. SML or underlying water column) or system specific, indicating that microenvironment physical and/or geochemical controls must be involved.

Table 4.1 Aqueous Fe(II) production (± standard error) from solid Fe(III) in microcosms inoculated with soluble Fe(III)-reducing enrichment communities (n=3) (t=end) from the surface microlayer (SML) and 0.5 m depth at Coldspring Lake (C) and Sunnyside Beach (S) in consecutive years relative to abiotic controls and adjusted for cell growth, and corresponding *in situ* large (>500 μm^2) floc abundance at the time of enrichment sampling.

Site, year, and depth	**S 2010 SML**	**S 2010 0.5 m**	**S 2011 SML**	**S 2011 0.5 m**	**C 2010 SML**	**C 2010 0.5 m**	**C 2011 SML**	**C 2011 0.5 m**
Fe(II) production (mM / A_{480})	11.8 ± 0.2 #*	0.9 ± 0.1 #	3.0 ± 0.1 #*	2.6 ± 0.0 #	0.0 *	0.3 ± 0.0	0.0 *	0.6 ± 0.1
Floc abundance (x10^2 flocs per mL)	7.4	0.0	-	-	0.2	2.3	-	-

Significantly different ($P < 0.05$) between systems for the same depth and year.

* Significantly different ($P < 0.05$) between depths for the same system and year.

For both lakes, consistent with the presence of a geochemically distinct SML that would appear more amenable to microbial Fe(III) reduction compared to underlying waters, aqueous SML concentrations of total Fe and PO_4^{3-} were higher ($P < 0.05$) compared to 0.5 m depth at the time of enrichment sampling (Table 4.2). The increased availability of PO_4^{3-} was expected to promote the heterotrophic formation of O_2-depleted floc microhabitats suitable for this metabolism (Paerl and Pinckney 1996). However, the capacity for soluble and solid Fe(III) reduction was not limited to the SML in upper waters, and solid Fe(III) reduction was absent only among enriched Coldspring Lake SML bacteria. Further, although Coldspring Lake had lower bulk O_2 saturation (95-100% compared to >105% at Sunnyside Beach), higher DOC concentrations (Drudge and Warren 2014), and higher ($P < 0.05$) total SML and 0.5 m depth concentrations of aqueous Fe and PO_4^{3-} (2.8-4.7 μM Fe and 0.06-0.10 mg/L

PO_4^{3-} compared to 1.0-2.4 μM Fe and 0.04-0.07 mg/L PO_4^{3-} at Sunnyside Beach) (Table 4.2), suggesting greater amenability to microbial Fe(III) reduction, Coldspring Lake SML soluble Fe(III)-reducing enrichment communities lacked members capable of reducing solid Fe(III). In addition, the production of Fe(II) by Coldspring Lake 0.5 m depth solid Fe(III)-reducing enrichment communities from both years was significantly ($P < 0.05$) lower than that observed for Sunnyside Beach enrichments from both depths (Table 4.1) despite significantly ($P < 0.05$) higher *in situ* total Fe concentrations at Coldspring Lake 0.5 m depth (2.8 ± 0.2 μM) compared to 0.5 m depth at Sunnyside Beach (1.0 ± 0.4 μM) and a similar concentration in the Sunnyside Beach SML (2.4 ± 0.9 μM) (Table 4.2). These results indicate that differences between lakes and depths with respect to the availability of total Fe and PO_4^{3-}, as well as O_2 levels and DOC concentrations, are not important controls on the solid Fe(III)-reducing capacity of bacteria at these locations.

Table 4.2 Mean (± standard error) concentrations of Fe (total and <0.2 μm) and PO_4^{3-} (total) in water samples collected from the surface microlayer (SML) and 0.5 m depth at Coldspring Lake and Sunnyside Beach in consecutive years (2010 and 2011) in the morning (n=6).

Parameter	**Coldspring Lake**		**Sunnyside Beach**	
	SML	**0.5 m depth**	**SML**	**0.5 m depth**
Fe (total) (μM)	4.7 ± 1.2#*	2.8 ± 0.2#	2.4 ± 0.9*	1.0 ± 0.4
Fe (<0.2 μm) (μM)	1.9 ± 0.4#*	0.9 ± 0.3	0.5 ± 0.2	0.4 ± 0.2
Fe (<0.2 μm) / Fe (>0.2 μm)	0.7	0.5	0.3	0.7
PO_4^{3-} (total) (mg/L)	0.10 ± 0.01#*	0.06 ± 0.01#	0.07 ± 0.02*	0.04 ± 0.01

Significantly different ($P < 0.05$) between systems at the same water depth.

* Significantly different ($P < 0.05$) within a system between water depths.

Results identify that the solid Fe(III)-reducing ability of Fe(III)-reducing bacteria appears to be inversely proportional to the availability of soluble Fe(III) rather than being influenced by total Fe availability. Corresponding with the absence of solid Fe(III)-reducing bacteria from Coldspring Lake SML enrichment communities from both years, dissolved (<0.2 μm) Fe concentrations at this lake and depth were significantly higher ($P < 0.05$) (1.9 ± 0.4 μM) compared to those at the 0.5 m depth (0.9 ± 0.3 μM) and both depths (0.5 ± 0.2 μM and 0.4 ± 0.2 μM for the SML and 0.5 m depth, respectively) at Sunnyside Beach (Table 4.2). The ratio of dissolved to particulate Fe was the same for the Coldspring Lake SML, from which solid Fe(III)-reducing bacteria were not enriched, and 0.5 m depth at Sunnyside Beach, enrichments which did include solid Fe(III) reducers, indicating that the absolute concentration of dissolved Fe, as opposed to its relative availability, was an important determinant of solid Fe(III) reducing capacity (between lakes and depths) (Table 4.2). The increased availability of dissolved Fe is consistent with the selection of Coldspring SML bacterial communities better adapted to reducing soluble Fe(III) rather than solid Fe(III) minerals, even if the latter are present dominantly as amorphous and/or biogenic, and thus more available forms (Zachara et al. 1998; Roden and Urrutia 2002; Langley et al. 2009). Of both lakes and water column depths, the Coldspring Lake SML is expected to have the highest soluble Fe(III) concentrations, as Coldspring Lake is highly organic- and Fe-rich relative to Sunnyside Beach and the SML of freshwater lakes is typically enriched in organic matter and specifically smaller sized organic molecules (Södergren 1993; Baastrup-Spohr and Staehr 2009; Hörtnagl et al. 2010), which can form soluble complexes with Fe(III). In addition, the Coldspring Lake SML was significantly ($P < 0.05$) enriched in PO_4^{3-}, which can form soluble complexes with Fe(III), relative to 0.5 m depth as well as both depths at

Sunnyside Beach (Table 4.2). Interestingly, Fe(II)-oxidizing bacteria, which are known to inhabit suspended flocs in oxic freshwaters where they closely interact with Fe(III) reducers (Elliott et al. 2014), appeared to be a major component of the 2011 Coldspring Lake SML enrichment community (one 97% sequence identity match constituting >25% of clone library sequences) (Figure 4.1). Putative Fe(II)-oxidizing bacteria were also previously detected in the Coldspring Lake SML environmental community (Drudge and Warren 2014). It has been proposed that Fe(II)-oxidizing bacteria produce chelators to bind soluble Fe(III) and transport it away from cell surfaces to avoid cell encrustation (Sobolev and Roden 2001), such that their presence in the Coldspring Lake SML could have increased the local availability of soluble Fe(III) for Fe(III)-reducing bacteria. Further, several bacteria capable of producing extracellular Fe(III)-solubilizing siderophores, namely *Achromobacter denitrificans* (Nishio et al. 1988), *Pseudomonas aeruginosa* (Poole and McKay 2003), and *Stenotrophomonas maltophilia* (García et al. 2012), closely matched (>98% sequence identity) sequences in clone libraries from Coldspring SML enrichment communities (Figure 4.1), suggesting that siderophore production also increased the availability of soluble Fe(III) at this location.

The midpoint potential of the Fe(III) citrate/Fe^{2+} redox couple is more positive compared to that for poorly crystalline Fe(III) oxide/Fe^{2+} (Mehta et al. 2005) and Fe(III)-reducing bacteria typically grow more slowly with solid Fe(III) than with soluble Fe(III) (Lovley 2013), implicating the increased availability of soluble Fe(III) as a driver for the selective absence of solid Fe(III)-reducing bacteria in the Coldspring Lake SML as they are outcompeted by the soluble Fe(III) reducers. Further, the more widespread presence of soluble versus solid Fe(III)-reducing bacteria across lakes and depths may be reflective of the increased accessibility and energetic potency of soluble Fe(III). Thus the absolute availability

of soluble Fe(III), linked to the presence of bacteria capable of oxidizing Fe(II) or producing siderophores as well as increased PO_4^{3-} availability, appears to be an important determinant of whether bacteria capable of solid Fe(III) reduction occur within freshwater littoral environments.

Besides the availability of soluble Fe(III) potentially determining the presence of solid Fe(III)-reducing bacteria, the physical structuring of bacterial communities may also influence their capacity for solid Fe(III) reduction through the creation of stable anoxic microenvironments suited to microbial solid Fe(III) reduction within larger flocs. In particular, the magnitude of Fe(II) production from solid Fe(III) by enrichment communities directly corresponded to the abundance of large (>500 μm^2) flocs between depths in each lake at the time of enrichment sampling in 2010 (Table 4.1), indicating that within each littoral site the relative availability of suitable low O_2 floc-based microhabitats was an important control on the presence and/or capacity of solid Fe(III)-reducing bacteria between depths. Based on Fe(III) reduction studies with *Geobacter* species, bacterial reduction of solid Fe(III) requires additional electron transfer proteins compared to soluble Fe(III) citrate reduction (Mehta et al. 2005; Ding et al. 2008; Smith et al. 2013), and enhanced expression of one of these proteins was found to be associated with more rapid aggregate formation (Summers et al. 2010). The dense microbial structuring of flocs would also facilitate close contact between anaerobic Fe(III) reducers and solid Fe(III) sorbed or aggregated within the floc matrix, which is otherwise expected to be limited in the ambient oxic water column. Fe(III) oxides are a key floc component and are closely associated with microbial cells in flocs (Liss et al. 1996; Plach et al. 2011; Elliott et al. 2012), providing a readily accessible source of solid Fe(III)-minerals for Fe(III)-reducing bacteria capable of using this substrate.

Consistent with a role for the dense microbial structuring of floc in facilitating solid Fe(III) reduction, the capacity for solid Fe(III) reduction by 0.5 m depth enrichment communities from Coldspring Lake, absent from corresponding Coldspring Lake SML enrichments, was associated with the presence of comparatively densely packed biofilms in microcosms in both years (Figure S.4.2).

The absence of enriched solid Fe(III)-reducing bacteria from the Coldspring SML may also have been a consequence of evaluating solid Fe(III)-reducing capacity using soluble Fe(III)-reducing enrichment communities instead of directly enriching for solid Fe(III)-reducing bacteria, thus excluding the examination of any bacteria able to reduce solid Fe(III) but not soluble Fe(III). However, the presence of solid Fe(III)-reducing bacteria in soluble Fe(III)-reducing enrichment communities from three of the four lake-depth combinations that were sampled in consecutive years suggests that conditions specifically present in the Coldspring SML were selecting for soluble Fe(III) reducers. Further, the absence of *Geobacter* spp. and other sedimentary Fe(III)-reducing bacteria dominantly reliant on solid Fe(III) in all enrichment communities emphasizes the clear ecological differentiation between anoxic sediments and the overlying oxic littoral water column, with the latter inhabited by flexible Fe(III) reducers capable of using more ecologically favorable soluble Fe(III). Although not constrained by this study, the specific measurement of soluble Fe(III) phases and their production by Fe(II)-oxidizing and siderophore-producing bacteria in the Coldspring SML would have clarified the role of soluble Fe(III) availability as a control on the absence of enriched solid Fe(III)-reducing bacteria from this location.

Here, results identify a diverse and specifically adapted population of soluble Fe(III)-reducing bacteria inhabiting the SML and underlying oxic water column in littoral regions of

two physically and geochemically contrasting freshwater lakes. These bacteria differed from classic sedimentary Fe(III) reducers, being adapted to high complex organic carbon availability and floc-related variable O_2 conditions present in the upper oxic water column of lakes. The absence of solid Fe(III)-reducing capacity among bacteria enriched from the SML at Coldspring Lake in consecutive years, unlike the SML at Sunnyside Beach or at 0.5 m depth in either lake, was linked to the higher concentration of soluble Fe(III) at this location, with the availability of large (>500 μm^2) flocs also appearing to directly influence the solid Fe(III) reduction capacity of enrichment communities between depths in each lake.

4.5 Supplemental Information

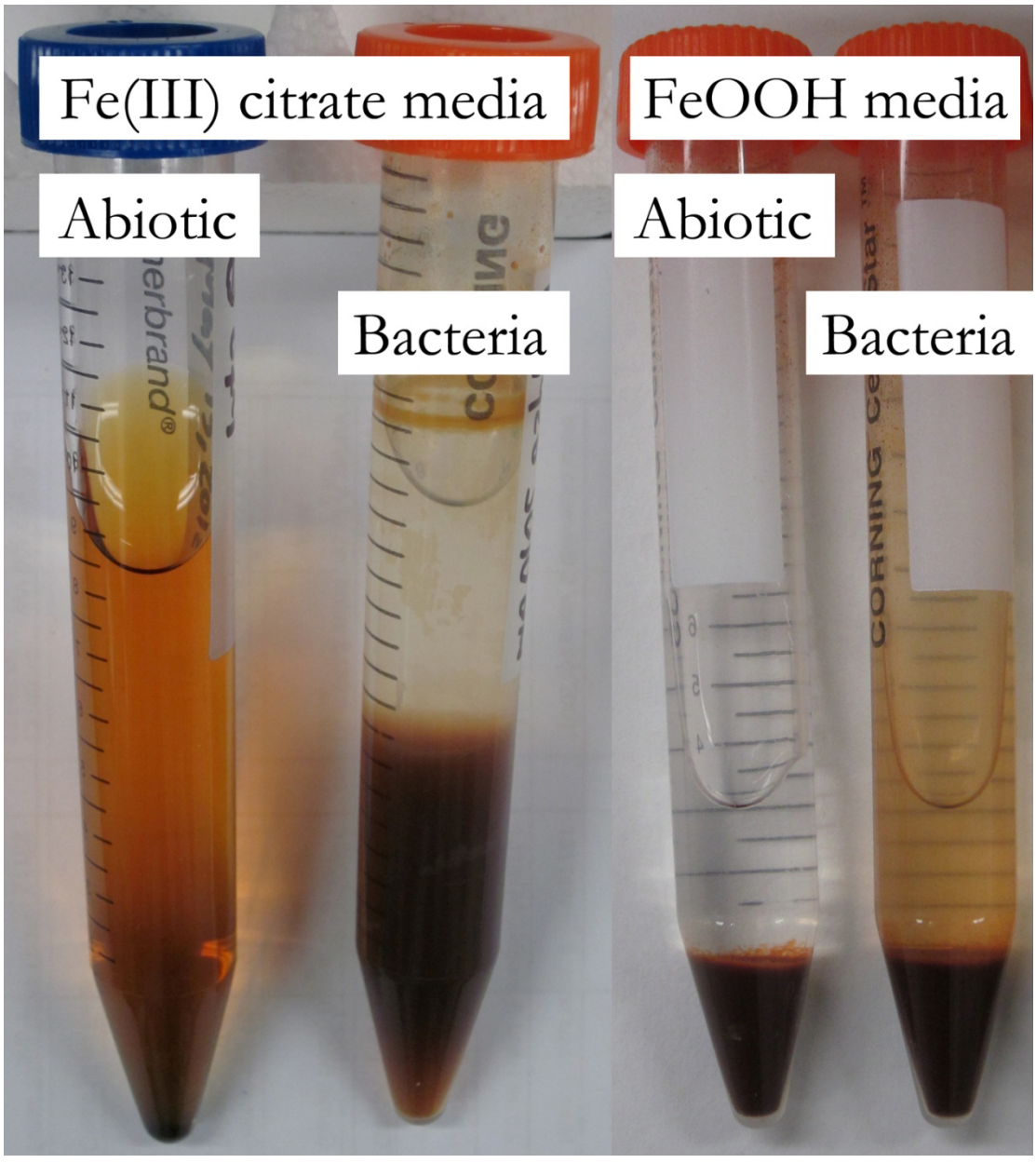

Figure S.4.1 Visual indicators of soluble and solid Fe(III) reduction by bacteria in microcosms containing soluble Fe(III) citrate- and solid FeOOH-based media, respectively.

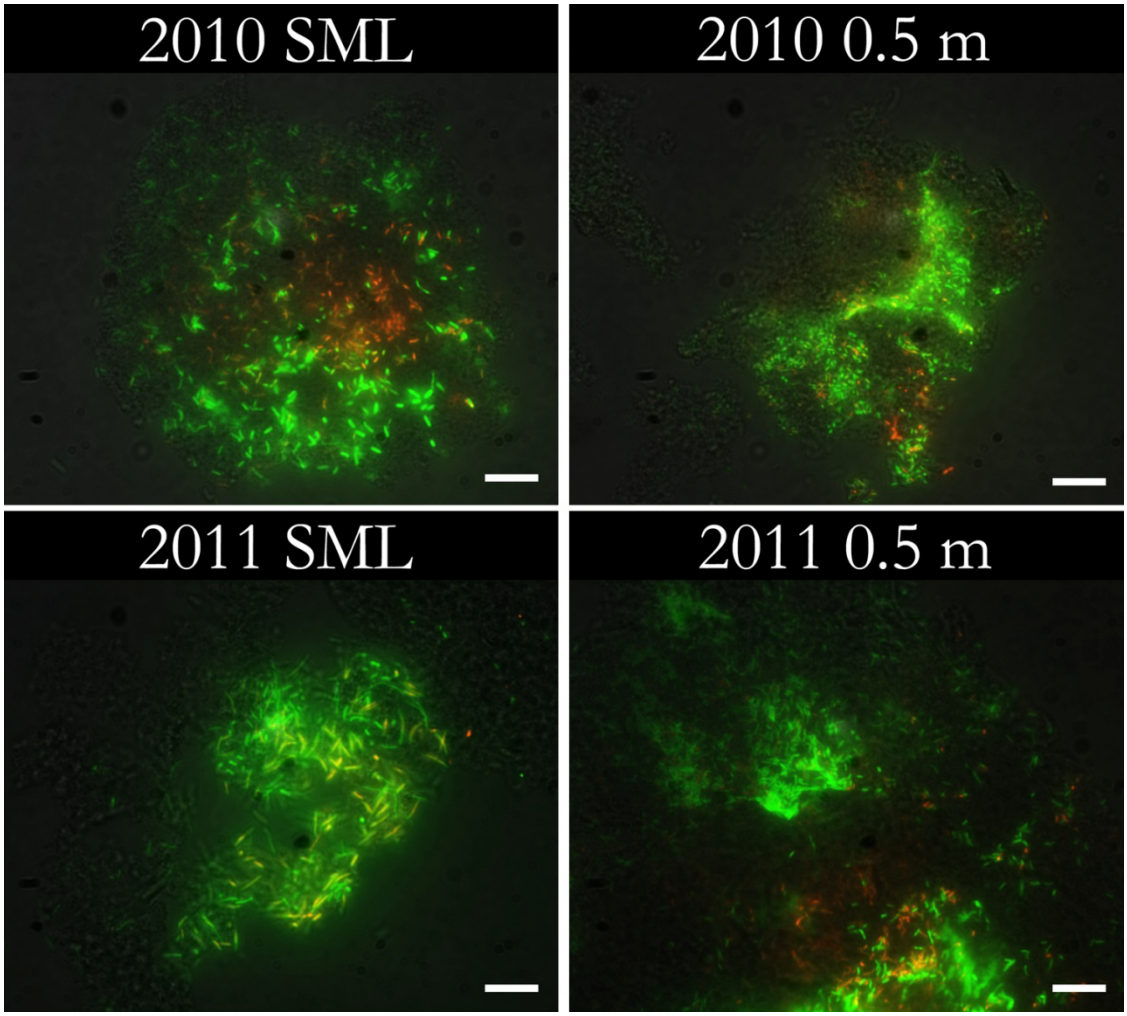

Figure S.4.2 Viable (green) and nonviable (red) bacterial cells (LIVE/DEAD staining) in microcosms (t=end) containing soluble Fe(III)-reducing enrichment communities sampled from the surface microlayer (SML) and at 0.5 m depth at Coldspring Lake in 2010 and 2011. Bars represent 10 μm.

4.6 References

Abramoff MD, Magelhaes PJ, Ram SJ. 2004. Image processing with ImageJ. Biophotonics International 11:36–42.

Allen TD, Lawson PA, Collins MD, Falsen E, Tanner RS. 2006. *Cloacibacterium normanense* gen. nov., sp. nov., a novel bacterium in the family *Flavobacteriaceae* isolated from municipal wastewater. Int J Syst Evol Microbiol 56(Pt 6):1311–1316.

Arfi R, Bouvy M. 1995. Size, composition and distribution of particles related to wind induced resuspension in a shallow tropical lagoon. J Plankton Res 17(3):557–574.

Armstrong DE, Elzerman AW. 1982. Trace metal accumulation in surface microlayers. J Great Lakes Res 8(2):282–287.

Baastrup-Spohr L, Staehr PA. 2009. Surface microlayers on temperate lowland lakes. Hydrobiologia 625(1):43–59.

Ballantyne AP, Brahney J, Fernandez D, Lawrence CL, Saros J, Neff JC. 2011. Biogeochemical response of alpine lakes to a recent increase in dust deposition in the Southwestern, US. Biogeosciences 8:2689–2706.

Balzano S, Statham PJ, Pancost RD, Lloyd JR. 2009. Role of microbial populations in the release of reduced iron to the water column from marine aggregates. Aquat Microb Ecol 54(3):291–303.

Boyd PW, Mackie DS, Hunter KA. 2010. Aerosol iron deposition to the surface ocean — Modes of iron supply and biological responses. Mar Chem 120(1–4):128–134.

Chan CS, Fakra SC, Edwards DC, Emerson D, Banfield JF. 2009. Iron oxyhydroxide mineralization on microbial extracellular polysaccharides. Geochim Cosmochim Acta 73(13):3807–3818.

Coates JD, Phillips EJ, Lonergan DJ, Jenter H, Lovley DR. 1996. Isolation of *Geobacter* species from diverse sedimentary environments. Appl Environ Microbiol 62(5):1531–1536.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res (37 Database):D141–145.

Crabill C, Donald R, Snelling J, Foust R, Southam G. 1999. The impact of sediment fecal coliform reservoirs on seasonal water quality in Oak Creek, Arizona. Water Res 33(9):2163–2171.

Crawford RL, Johnson L, Martinson M. 1982. Bacterial enrichments in surface films of freshwater lakes. J Great Lakes Res 8(2):323–325.

Cunliffe M, Murrell JC. 2009. The sea-surface microlayer is a gelatinous biofilm. ISME J 3(9):1001–1003.

Cunliffe M, Harrison E, Salter M, Schafer H, Upstill-Goddard RC, Murrell JC. 2009. Comparison and validation of sampling strategies for the molecular microbial ecological analysis of surface microlayers. Aquat Microb Ecol 57(1):69–77.

Cunliffe M, Upstill-Goddard RC, Murrell JC. 2011. Microbiology of aquatic surface microlayers. FEMS Microbiol Rev 35(2):233–246.

Cuong DT, Karuppiah S, Obbard JP. 2008. Distribution of heavy metals in the dissolved and suspended phase of the sea-surface microlayer, seawater column and in sediments of Singapore's coastal environment. Environ Monit Assess 138(1–3):255–272.

Ding YH, Hixson KK, Aklujkar MA, Lipton MS, Smith RD, Lovley DR, Mester T. 2008. Proteome of *Geobacter sulfurreducens* grown with Fe(III) oxide or Fe(III) citrate as the electron acceptor. Biochim Biophys Acta 1784(12):1935–1941.

Dong D, Nelson YM, Lion LW, Shuler ML, Ghiorse WC. 2000. Adsorption of Pb and Cd onto metal oxides and organic material in natural surface coatings as determined by selective extractions: new evidence for the importance of Mn and Fe oxides. Water Res 34(2):427–436.

Droppo IG. 2001. Rethinking what constitutes suspended sediment. Hydrol Processes 15(9):1551–1564.

Droppo IG, Irvine KN, Jaskot C. 2002. Flocculation/aggregation of cohesive sediments in the urban continuum: implications for stormwater management. Environ Technol 23(1):27–41.

Drudge CN and Warren LA. 2014. Diurnal floc generation from neuston biofilms in two contrasting freshwater lakes. Environ Sci Technol 48(17):10107–10115.

Elliott AVC, Plach JM, Droppo IG, Warren LA. 2012. Comparative floc-bed sediment trace element partitioning across variably contaminated aquatic ecosystems. Environ Sci Technol 46(1):209–216.

Elliott AVC, Warren LA. 2014. Microbial engineering of floc Fe and trace element geochemistry in a circumneutral, remote lake. Environ Sci Technol 48(12):6578–6587.

Elliott AVC, Plach JM, Droppo IG, Warren LA. 2014. Collaborative microbial Fe-redox cycling by pelagic floc bacteria across wide ranging oxygenated aquatic systems. Chem Geol 366:90–102.

Ellwood MJ, Nodder SD, King AL, Hutchins DA, Wilhelm SW, Boyd PW. 2014. Pelagic iron cycling during the subtropical spring bloom, east of New Zealand. Mar Chem 160:18–33.

Falkowska L. 1999. Sea surface microlayer: a field evaluation of teflon plate, glass plate and screen sampling techniques. Part 2. Dissolved and suspended matter. Oceanologia 41:223–240.

Ferris FG. 2005. Biogeochemical properties of bacteriogenic iron oxides. Geomicrobiol J 22(3–4):79–85.

Franklin MP, McDonald IR, Bourne DG, Owens NJ, Upstill-Goddard RC, Murrell JC. 2005. Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. Environ Microbiol 7(5):723–736.

García CA, Passerini De Rossi B, Alcaraz E, Vay C, Franco M. 2012. Siderophores of *Stenotrophomonas maltophilia*: detection and determination of their chemical nature. Rev Argent Microbiol 44(3):150–154.

García-Balboa C, Vicente MS, Blázquez ML, González F, Muñoz JA, Ballester A. 2011. Iron speciation in dissimilatory Fe(III)-reducing cultures. Geomicrobiol J 28(4):371–379.

Gault AG, Ibrahim A, Langley S, Renaud R, Takahashi Y, Boothman C, Lloyd JR, Clark ID, Ferris FG, Fortin D. 2011. Microbial and geochemical features suggest iron redox cycling within bacteriogenic iron oxide-rich sediments. Chem Geol 281(1–2):41–51.

Hardy JT, Coley JA, Antrim LD, Kiesser SL. 1988. A hydrophobic large-volume sampler for collecting aquatic surface microlayers: characterization and comparison with the glass plate method. Can J Fish Aquat Sci 45(5):822–826.

Hatzinger PB, Palmer P, Smith RL, Peñarrieta CT, Yoshinari T. 2003. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. J Microbiol Methods 52(1):47–58.

Hörtnagl P, Pérez MT, Sommaruga R. 2010. Living at the border: A community and single-cell assessment of lake bacterioneuston activity. Limnol Oceanogr 55(3):1134–1144.

Ju X, Jin Y, Sasaki K, Saito N. 2008. Perfluorinated surfactants in surface, subsurface water and microlayer from Dalian Coastal waters in China. Environ Sci Technol 42(10):3538–3542.

Kim SJ, Park SJ, Oh YS, Lee SA, Shin KS, Roh DH, Rhee SK. 2012. *Shewanella arctica* sp. nov., an iron-reducing bacterium isolated from Arctic marine sediment. Int J Syst Evol Microbiol 62(Pt 5):1128–1133.

Kjelleberg S, Stenström TA, Odham G. 1979. Comparative study of different hydrophobic devices for sampling lipid surface films and adherent microorganisms. Mar Biol 53(1):21–25.

Kostka J, Nealson KH. 1998. Isolation, cultivation and characterization of iron-and manganese-reducing bacteria. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G, editors. Techniques in Microbial Ecology. New York: Oxford University Press. P 58–78.

Kühl M, Glud RN, Ploug H, Ramsing NB. 1996. Microenvironmental control of photosynthesis and photosynthesis-coupled respiration in an epilithic cyanobacterial biofilm. J Phycol 32(5):799–812.

Langley S, Gault A, Ibrahim A, Renaud R, Fortin D, Clark ID, Ferris FG. 2009. A comparison of the rates of Fe (III) reduction in synthetic and bacteriogenic iron oxides by *Shewanella putrefaciens* CN32. Geomicrobiol J 26(2):57–70.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23(21):2947–2948.

Lehours AC, Batisson I, Guedon A, Mailhot G, Fonty G. 2009. Diversity of culturable bacteria, from the anaerobic zone of the meromictic Lake Pavin, able to perform dissimilatory-iron reduction in different in vitro conditions. Geomicrobiol J 26(3):212–223.

Lentini CJ, Wankel SD, Hansel CM. 2012. Enriched iron(III)-reducing bacterial communities are shaped by carbon substrate and iron oxide mineralogy. Front Microbiol 3:404.

Liss SN, Droppo IG, Flannigan DT, Leppard GG. 1996. Floc architecture in wastewater and natural riverine systems. Environ Sci Technol 30(2):680–686.

Logan BE. 2009. Exoelectrogenic bacteria that power microbial fuel cells. Nat Rev Microbiol 7(5):375–381.

Loge FJ, Emerick RW, Thompson DE, Nelson DC, Darby JL. 1999. Factors influencing ultraviolet disinfection performance Part I: Light penetration to wastewater particles. Water Environ Res 71(3):377–381.

Lovley DR, Phillips EJ. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54(6):1472–1480.

Lovley DR. 2013. Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes. New York: Springer. P 287–308.

Lu S, Chourey K, Reiche M, Nietzsche S, Shah MB, Neu TR, Hettich RL, Küsel K. 2013. Insights into the structure and metabolic function of microbes that shape pelagic iron-rich aggregates ("iron snow"). Appl Environ Microbiol 79(14):4272–4281.

Mahowald NM, Engelstaedter S, Luo C, Sealy A, Artaxo P, Benitez-Nelson C, Bonnet S, Chen Y, Chuang PY, Cohen DD, Dulac F, Herut B, Johansen AM, Kubilay N, Losno R, Maenhaut W, Paytan A, Prospero JM, Shank LM, Siefert RL. 2009. Atmospheric iron deposition: global distribution, variability, and human perturbations. Annu Rev Mar Sci 1:245–278.

Mehta T, Coppi MV, Childers SE, Lovley DR. 2005. Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. Appl Environ Microbiol 71(12):8634–8641.

Michelbach S. 1995. Origin, resuspension and settling characteristics of solids transported in combined sewage. Water Sci Technol 31(7):69–76.

Münster U, Heikkinen E, Knulst J. 1997. Nutrient composition, microbial biomass and activity at the air-water interface of small boreal forest lakes. Hydrobiologia 363(1-3):261–270.

Myers CR, Nealson KH. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science 240(4857):1319–1321.

Nevin KP, Holmes DE, Woodard TL, Hinlein ES, Ostendorf DW, Lovley DR. 2005. *Geobacter bemidjiensis* sp. nov. and *Geobacter psychrophilus* sp. nov., two novel Fe(III)-reducing subsurface isolates. Int J Syst Evol Microbiol 55(Pt 4):1667–1674.

Newton RJ, Jones SE, Eiler A, McMahon KD, Bertilsson S. 2011. A guide to the natural history of freshwater lake bacteria. Microbiol Mol Biol Rev 75(1):14–49.

Nishio T, Tanaka N, Hiratake J, Katsube Y, Ishida Y, Oda J. 1988. Isolation and structure of the novel dihydroxamate siderophore alcaligin. J Am Chem Soc 110(26):8733–8734.

Paerl HW, Pinckney JL. 1996. A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. Microb Ecol 31(3):225–247.

Plach JM, Elliott AV, Droppo IG, Warren LA. 2011. Physical and ecological controls on freshwater floc trace metal dynamics. Environ Sci Technol 45(6):2157–2164.

Poole K, McKay GA. 2003. Iron acquisition and its control in *Pseudomonas aeruginosa*: many roads lead to Rome. Front Biosci 8:d661–686.

Prakash O, Gihring TM, Dalton DD, Chin KJ, Green SJ, Akob DM, Wanger G, Kostka JE. 2010. *Geobacter daltonii* sp. nov., an Fe(III)- and uranium(VI)-reducing bacterium isolated from a shallow subsurface exposed to mixed heavy metal and hydrocarbon contamination. Int J Syst Evol Microbiol 60(Pt 3):546–553.

Reinthaler T, Sintes E, Herndl GJ. 2008. Dissolved organic matter and bacterial production and respiration in the sea-surface microlayer of the open Atlantic and the western Mediterranean Sea. Limnol Oceanogr 53(1):122–136.

Roden EE, Urrutia MM. 2002. Influence of biogenic Fe (II) on bacterial crystalline Fe (III) oxide reduction. Geomicrobiol J 19(2):209–251.

Schwertmann U, Cornell RM. 2000. Iron Oxides in the Laboratory. New York: Wiley-VHC. 188 p.

Shanks AL, Reeder ML. 1993. Reducing microzones and sulfide production in marine snow. Mar Ecol Prog Ser 96:43–47.

Simon M, Grossart HP, Schweitzer B, Ploug H. 2002. Microbial ecology of organic particles in aquatic ecosystems. Aquat Microb Ecol 28(2):175–211.

Smith JA, Lovley DR, Tremblay PL. 2013. Outer cell surface components essential for Fe(III) oxide reduction by *Geobacter metallireducens*. Appl Environ Microbiol 79(3):901–907.

Sobolev D, Roden EE. 2001. Suboxic deposition of ferric iron by bacteria in opposing gradients of Fe(II) and oxygen at circumneutral pH. Appl Environ Microbiol 67(3):1328–1334.

Södergren A. 1993. Role of aquatic surface microlayer in the dynamics of nutrients and organic compounds in lakes, with implications for their ecotones. Hydrobiologia 251(1–3):217–225.

Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, Lovley DR. 2010. Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. Science 330(6009):1413–1415.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30(12):2725–2729.

Venkateswaran K, Dollhopf ME, Aller R, Stackebrandt E, Nealson KH. 1998. *Shewanella amazonensis* sp. nov., a novel metal-reducing facultative anaerobe from Amazonian shelf muds. Int J Syst Bacteriol 48(Pt 3):965–972.

Viollier E, Inglett PW, Hunter K, Roychoudhury AN, Van Cappellen P. 2000. The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. Appl Geochem 15(6):785–790.

Whitman RL, Nevers MB, Byappanahalli MN. 2006. Examination of the watershed-wide distribution of *Escherichia coli* along Southern Lake Michigan: an integrated approach. Appl Environ Microbiol 72(11):7301–7310.

Wurl O, Obbard JP. 2004. A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. Mar Pollut Bull 48(11–12):1016–1030.

Zachara JM, Fredrickson JK, Li SM, Kennedy DW, Smith SC, Gassman PL. 1998. Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. Amer Mineral 83(11–12 Pt 2):1426–1443.

Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F, Han SK. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol 28(2):141–155.

CHAPTER 5: SELECTIVE PROMOTION OF ANTIBIOTIC RESISTANCE IN FE(III)-REDUCING MEMBERS OF FRESHWATER SURFACE MICROLAYER BACTERIAL COMMUNITIES

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5.1 Abstract

Environmental bacterial communities comprise diverse metabolic guilds that may be differentially exposed to trace metals, e.g. Fe(III)-reducing bacteria exposed to liberated metals associated with their reductive dissolution of Fe oxyhydroxides, and thus may exhibit varying levels of metal and antibiotic resistance. However, results here demonstrated inhibition of soluble Fe(III) reduction at significantly (p<0.05) lower concentrations of trace metal or antibiotic cocktails compared to inhibition of bulk community metabolic activity for bacterial communities enriched from the surface microlayer (SML) and underlying waters (0.5 m depth) of two contrasting lake sites. Further, Cu exposure selectively inhibited Fe(II) production by solid Fe(III)-reducing enrichment community members and was associated with a decrease in the relative abundance of known Fe(III)-reducing bacterial genera (*Aeromonas* and *Clostridium*) in 16S rRNA gene clone libraries derived from enrichments. Interestingly, unlike trace metal resistance, antibiotic resistance was significantly (p<0.05) higher among soluble Fe(III)-reducing members of SML enrichment communities compared to those from 0.5 m depth, pointing to the selective promotion of antibiotic resistance in SML communities independent of metal and antibiotic resistance co-selection mechanisms. This finding, along with the detection of mobile genetic elements and potential opportunistic

pathogens in enrichment communities, identifies the SML as a possible reservoir of health-relevant bacteria within aquatic systems.

5.2 Introduction

Resistance to trace metals and antibiotics can vary substantially among members of environmental bacterial communities, such that the presence of these stressors can selectively inhibit specific metabolic activities [1, 2] and favor the growth of specific metabolic guilds [3, 4]. In freshwater environments, the documented importance of Fe(III) oxyhydroxides (FeOOH) for trace metal sequestration [5-7], suggests that bacteria able to reductively dissolve these minerals and thereby increase their exposure to metals are characteristically more metal resistant. Bacteria can appreciably reduce extracellular Fe(III) by using it as a terminal electron acceptor or electron sink, or by producing assimilatory reductase enzymes [8, 9].

In well-oxygenated circumneutral freshwater environments, suspended flocs enable Fe(III)-reducing bacteria to occupy O_2-depleted zones where they can readily access fresh Fe(III) sources, particularly highly metal rich amorphous/labile phases, which can otherwise be scarce in anoxic environments [10]. Relative to their neighbors within flocs and other aquatic biofilm communities, Fe(III)-reducing bacteria are likely more highly exposed to trace metals due to their close proximity to, and extremely localized reductive dissolution of trace metal-sequestering Fe(III) minerals [11, 12]. Enhanced metal stress among Fe(III) reducers in this setting could selectively increase their acquisition and maintenance of metal resistance mechanisms, resulting in higher metal resistance within this metabolic guild compared to other non-Fe(III)-reducing bacteria. Further, the potential co-selection of trace

metal and antibiotic resistance in bacteria via shared resistance mechanisms or genetic linkage of resistance determinants in mobile elements [13, 14] indicates that antibiotic resistance is also relatively higher among Fe(III) reducers in aquatic bacterial communities.

The surface microlayer (SML), a distinct bacterial habitat occupying the topmost millimeter of the water column, is an accumulation site for organic matter, nutrients, and Fe, the substrates necessary to support bacterial Fe(III) reduction [15-17]. The SML has been shown to generate and accumulate dense microbial communities in the form of flocs and precursor biofilms [17, 18] that in addition to providing a Fe(III)-enriched habitat for Fe(III)-reducing bacteria are expected to be highly antagonistic and include antibiotic-producing species [19, 20]. Further, high microbial density can facilitate horizontal gene transfer between bacteria via mobile genetic elements, promoting the acquisition and dissemination of genes conferring resistance to environmental stressors. The SML is also an accumulation site for toxic organic and inorganic contaminants including relatively toxic trace metals [21, 22]. These characteristics suggest that the SML is a crucible for the development of bacterial resistance to trace metals and antibiotics. Supporting this notion, higher levels of resistance to multiple classes of antibiotics and a wide range of trace metals have been observed among bacteria cultured from the SML compared to underlying waters in both marine and freshwater environments [23-25]. However, the relative resistance of Fe(III) reducers within environmental bacterial communities, including the SML community, has not been investigated to date.

Given the putative links between Fe(III)-reducing bacteria, trace metal exposure, and resistance to metals and antibiotics, as well as the ecological distinctiveness of the SML, the objectives of this study were to examine the resistance of Fe(III)-reducing bacteria within

mixed Fe(III)-reducing communities enriched from the SML and underlying water column (0.5 m depth) of two contrasting freshwater sites. Specifically, the objectives were to examine these bacteria with respect to (1) their relative trace metal and antibiotic resistance compared to non-Fe(III)-reducing members of individual enrichment communities, and (2) their relative health relevance between sites and depths, based on their level of antibiotic and trace metal resistance and inclusion of mobile genetic elements capable of disseminating resistance determinants.

5.3 Materials and Methods

5.3.1 Description of Sampling Sites

Soluble Fe(III)-reducing communities (n=7) were enriched directly in the field from water samples collected from the SML and underlying water column (0.5 m depth) at Sunnyside Beach and Coldspring Lake in consecutive summers (2010 and 2011) over a single one or two day excursion. Sunnyside Beach is a public beach on the northwestern shore of Lake Ontario (43°38'14"N 79°27'21"W) in downtown Toronto, Ontario, Canada (population ~3 million) that is impacted by wastewater effluents. In contrast, Coldspring Lake is a small (<1 km^2) highly organic and Fe-rich lake in a remote nature reserve zone in the northwestern region (45°51'12"N 78°49'24"W) of Algonquin Provincial Park (Ontario, Canada). At both sampling sites, the water column was not stratified and sampling depths were well oxygenated (>95% dissolved O_2 saturation) and within the shallow littoral zone (water column depth was <3 m at Coldspring Lake and <1 m at Sunnyside Beach, >1% of PAR irradiance reached the sediment-water interface at both sites) [17].

5.3.2 Enrichment of Soluble Fe(III)-Reducing Communities

Samples were collected from the SML using nucleation-track polycarbonate membrane filters (47 mm diameter, 0.1 μm pore size) as described by Cunliffe et al. [26]. At each sampling time, the SML was randomly collected within a ~1 m^2 area using five filters applied to the water surface, followed by the collection of water from 0.5 m depth using a Van Dorn bottle sampler. Filters (SML) or 350 μL aliquots (0.5 m depth; approximately the volume of a SML sample) were used to inoculate 13 mL of a soluble Fe(III) citrate-based Fe(III) reducer enrichment medium [27] in sterile 15 mL Falcon tubes (BD Biosciences). The medium included 10 mM Na acetate as a carbon substrate and 25 mM Fe(III) citrate as a source of soluble complexed Fe(III). In addition, 0.01% (w/v) yeast extract was included to promote heterotrophic growth and provide an additional source of organic carbon. Soluble Fe(III)-reducing bacteria were specifically targeted for enrichment because their clearing of orange-brown soluble Fe(III) citrate from solution and subsequent formation of a brown precipitate provides a clear and relatively rapid visual indicator of their activity [10, 27]. This enabled the selective assessment of the resistance of soluble Fe(III) reducers within mixed soluble Fe(III)-reducing enrichment communities.

Enrichment tubes were incubated at room temperature (~23°C) in the dark to mimic the attenuation of sunlight within flocs where bacterial Fe(III) reduction would be expected to occur in oxic waters [28]. This approach also minimized the impact of photochemical effects on the stability of Fe(III) citrate. Tubes were tightly capped and left stationary, resulting in the development of microoxic (<15% O_2 saturation) conditions within 48 h.

5.3.3 Determination of Metal and Antibiotic Resistance of Fe(III)-Reducing Bacteria Versus Other Members of Soluble Fe(III)-Reducing Enrichment Communities

A 96-well plate assay was employed to assess the trace metal and antibiotic resistance of soluble Fe(III)-reducing bacteria versus other members of soluble Fe(III)-reducing enrichment communities based on the inhibition of the metabolic activity of these two groups over a range of concentrations of trace metal or antibiotic cocktails (Figure S.5.1). Soluble Fe(III) reducer activity was visualized using the aforementioned soluble Fe(III) citrate-based enrichment medium (Figure S.5.1). Bulk enrichment community activity, encompassing both soluble Fe(III)-reducing and other non-Fe(III)-reducing enrichment community members, was visualized using nonselective YPG medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) that included 0.01% (w/v) iodonitrotetrazolium chloride (INT) (Sigma-Aldrich). INT is a colourless tetrazolium salt that is reduced to a coloured (violet) product by general bacterial respiration (Figure S.5.1) and has been previously used to assess the respiratory activity of environmental bacterial communities [29] and the antibiotic susceptibility of specific bacteria [30].

Using 96-well transparent PCR plates with well volumes of 200 μL (Bio-Rad), wells were filled with 175 μL of soluble Fe(III) citrate-based medium or YPG medium + 0.01% (w/v) INT, along with 20 μL of the metal or antibiotic cocktail diluted with phosphate buffered saline solution (1xPBS) to ensure the appropriate final cocktail concentration in the well. Wells were then inoculated with 5 μL of a soluble Fe(III)-reducing enrichment community, from each of the SML and underlying water (0.5 m depth) from both sites and years being evaluated. Eight replicate wells were tested per concentration of metal or antibiotic cocktail for each enrichment community. A positive control (growth medium and

enrichment inoculum) and two negative controls (growth medium only, medium and highest metal or antibiotic cocktail concentration) were used to ensure appropriate positive visual indications of the activity of soluble Fe(III)-reducing bacteria as well as bulk enrichment community activity. For each plate, visual metabolic activity indicators were observed after a 30 d incubation period. The assay was repeated if Fe(III) reducer or bulk enrichment community activity was not visually confirmed in all positive control wells.

Resistance to trace metals and antibiotics was evaluated using cocktails rather than individual compounds to better represent the exposure conditions for bacterial communities in natural aquatic environments. To assess trace metal resistance, soluble Fe(III)-reducing enrichment communities were exposed to increasing concentrations of a metal cocktail consisting of Ag^{+}, Cd^{2+}, CrO_4^{2-}, Pb^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} (all acetate salts) maintained at an absolute ratio of 1:1:1:1:5:5:10 (w/v). Four increasing cocktail concentrations were tested, corresponding to concentrations of 5, 10, 50, and 100 ng/mL Ag^{+}, Cd^{2+}, CrO_4^{2-}, and Pb^{2+}, 25, 50, 250, and 500 ng/mL Cu^{2+} and Ni^{2+}, and 50, 100, 500, and 1000 ng/mL Zn^{2+}. The metals used in the cocktail were selected because they collectively activate several distinct bacterial resistance mechanisms [31] and are common, often co-occurring contaminants in freshwater environments. The relative concentration of each metal in the cocktail was based on previously measured freshwater concentrations in Lake Ontario [32].

To assess antibiotic resistance, soluble Fe(III)-reducing enrichment communities were exposed to a 1:1:5:10:10 (w/v) cocktail of cephalexin, tetracycline, ciprofloxacin, erythromycin, and sulfamethoxazole. Eight cocktail concentrations were tested, corresponding to concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 μg/ml cephalexin and tetracycline, 0.25, 0.5, 2.5, 5, 25, 50, 250, and 500 μg/ml ciprofloxacin, and 0.5, 1, 5, 10, 50,

100, 500 μg/ml erythromycin and sulfamethoxazole. These antibiotics were selected because they collectively activate a wide range of bacterial resistance mechanisms [33] and have been previously detected in freshwater environments [34, 35]. The relative concentration of each antibiotic in the cocktail was based on previously measured freshwater concentrations [34].

Relative resistance (RR), an ordinal scale representing the trace metal or antibiotic cocktail concentration required to inhibit positive visual metabolic activity indicators (lower number = activity inhibited at a lower concentration, i.e. less resistant), was developed for this study. Inhibition of the activity of Fe(III)-reducing bacteria or bulk enrichment community activity for metal exposure was evaluated by a RR scale of 1 to 4. Bacterial inhibition associated with antibiotic exposure was evaluated by a RR scale of 1 to 8. A larger number of cocktail concentrations were tested for antibiotics compared to trace metals in order to clarify differences between bulk enrichment community activity and the activity of soluble Fe(III)-reducing community members that were less apparent based on preliminary experiments (not included) assessing fewer antibiotic cocktail concentrations.

5.3.4 Determination of Cu Resistance of Solid Fe(III)-Reducing Bacteria Versus Other Members of Soluble Fe(III)-Reducing Enrichment Communities

To confirm and expand on the results of the 96-well plate assay, a microcosm-based assay was carried out to determine the Cu resistance of solid Fe(III)-reducing bacteria compared to soluble Fe(III)-reducing enrichment community members that do not reduce solid Fe(III). Cu was selected as a representative to determine trace metal resistance because it is a common aquatic contaminant known to accumulate in the SML [21, 22] and has a high affinity for both organic matter and FeOOH [36, 37].

Microcosms were set up using 15 mL Falcon tubes filled with 13 mL of a growth medium based on a synthetic FeOOH mixture. The FeOOH mixture, intended to reproduce the dominant forms of solid Fe(III) found in circumneutral non-sulfidic freshwater environments, namely ferrihydrite and more amorphous Fe(III) solids, was prepared by neutralizing a solution of $FeCl_3$ with NaOH according to a method by Schwertmann and Cornell [38]. The medium consisted of 0.1% w/v tryptone, 0.02% w/v yeast extract, 0.3% w/v sodium acetate, and 15% v/v settled FeOOH mixture slurry (~50 mM Fe(III)).

To determine the relative Cu resistance of solid Fe(III)-reducing bacteria compared to other members of each soluble Fe(III)-reducing enrichment community (n=7), the activity of solid Fe(III) reducers and bulk community activity were measured in six microcosms inoculated with 1 mL of a previously established soluble Fe(III)-reducing enrichment community. Three of the microcosms were treated with 500 μM Cu acetate 48 h prior to inoculation to allow for Cu sorption to FeOOH. A high relative sorbent to sorbate ratio of ~50 mM solid Fe(III) to 500 μM Cu ensured near complete ~100% sorption of Cu to the FeOOH (t=0, solution [Cu] non-detectable). Thus, any inhibition of Fe(III) reduction associated with Cu exposure would reflect active reductive dissolution of the solid FeOOH and associated release of Cu into solution. Uninoculated tubes were used as a sterile abiotic control. The reduction of solid Fe(III), as an indicator of the activity of solid Fe(III)-reducing bacteria, was quantitatively assessed by the detection of Fe(II) at concentrations significantly (p<0.05) higher than abiotic controls. Fe(II) production was measured in microcosms at 72 to 120 h intervals over a 672 h incubation period. Fe(II) concentrations (n=3 per community) were measured according to the ferrozine method described by Viollier et al. [39] following agitation (inverting microcosm tubes several times), in order to detect

Fe(II) in aqueous solution as well as weakly associated with solids [40]. To minimize contamination, all equipment and containers used for the measurement of Fe(II) or solution Cu concentrations were soaked in 4% (v/v) HCl for >24 h and then rinsed eight times with ultrapure water (18.2 MΩ cm; Milli-Q; Millipore) before use. Concurrent with the measurement of Fe(II) production (i.e. metabolic activity of solid Fe(III)-reducing bacteria), bulk activity of the Fe(III)-reducing enrichment community was determined by diluting 15 μL aliquots from agitated microcosms in 135 μL (1:10 dilution) of YPG medium + 0.01% (w/v) INT as a colourimetric indicator of general bacterial respiration. Following a 48 h incubation period at room temperature (~23°C), these preparations were diluted 1:10 in ultrapure water and the absorbance of the resulting solution was measured at 480 nm, providing a rough measure of bulk community activity. At the end of the incubation period, the bacterial community composition of microcosms untreated or treated with 500 μM Cu was determined by 16S rRNA gene sequencing. Total community DNA was isolated from 450 μL of agitated microcosms (pooled 150 μL aliquots from the three replicate tubes) using the PowerSoil DNA Isolation Kit (MO Bio Laboratories) according to the manufacturer's instructions. Bacterial 16S rRNA genes were selectively amplified by PCR using the 27F/1492R primer set according to Zwart et al. [41], and then cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Between 25 and 30 clones containing inserts were sequenced (~1000 nucleotides) per enrichment (MOBIX Lab, McMaster University). Bacterial 16S rRNA gene sequences were submitted to the DDBJ/EMBL/GenBank databases and have been assigned accession numbers KM091641 to KM091674. The closest database matches to each sequence were obtained using BLAST (http://blast.ncbi.nlm.nih.gov/). Clone libraries derived from untreated microcosms for

both SML and 0.5 m depth Fe(III)-reducing enrichments consisted of a diverse group of bacteria that included facultative and obligate anaerobes and varied substantially between sites and depths (C. N. Drudge and L. A. Warren, submitted October 2014 to Geomicrobiology Journal).

5.3.5 PCR-Based Detection of Mobile Genetic Elements Associated with Resistance

Gene cassettes, consisting of a single gene and a recombination site, associated with class 1 integrons were selectively amplified from soluble Fe(III)-reducing enrichment community DNA (isolation method the same as described in the previous section for FeOOH-based microcosms) by PCR using the HS286/HS287 primer set targeting recombination sites that delimit individual cassettes [42]. Gel electrophoresis (1.8% w/v agarose gel) of gene cassette PCR products was carried out with use of a DNA ladder to determine the number and approximate size of PCR amplicons, products of the amplification of either a single cassette or multiple cassettes in an array. DNA sequences specific to particular plasmid types (*trfA2* for IncP-1 and *oriV* for IncQ) were also detected in enrichment community DNA by their selective PCR amplification according to Götz et al. [43]. Each PCR assay was performed in duplicate and the detection of elements reported only for consistently positive results.

5.3.6 Statistical Analysis

Statistical analysis was carried out using StatPlus:mac LE (version 2009; AnalystSoft, http://www.analystsoft.com). Statistical comparison of the resistance of (1) Fe(III)-reducing enrichment communities and specifically their Fe(III)-reducing members enriched from the

SML compared to from 0.5 m depth and (2) metabolically mixed parent Fe(III)-reducing enrichment communities from both depths compared to their Fe(III)-reducing members to a range of concentrations of cocktails of antibiotics or trace metals was evaluated using the paired sign test. One-way analysis of variance (ANOVA) was used to test for significant differences in mean Fe(II) concentrations between solid Fe(III)-reducing enrichment microcosms treated with Cu or left untreated, with individual paired comparisons subsequently being tested for significance using Tukey's HSD test. For all tests, $p<0.05$ was considered statistically significant.

5.4 Results

Based on visual indicators of metabolic activity, soluble Fe(III)-reducing bacteria were significantly ($p<0.05$) less resistant to cocktails of trace metals or antibiotics compared to the larger soluble Fe(III)-reducing enrichment communities they were part of, for enrichments recovered from the SML and at 0.5 m depth for both sites in consecutive years (Figure 5.1). The magnitude of metal and antibiotic resistance in soluble Fe(III)-reducing enrichment communities did not clearly differ between sites (Figure 5.1).

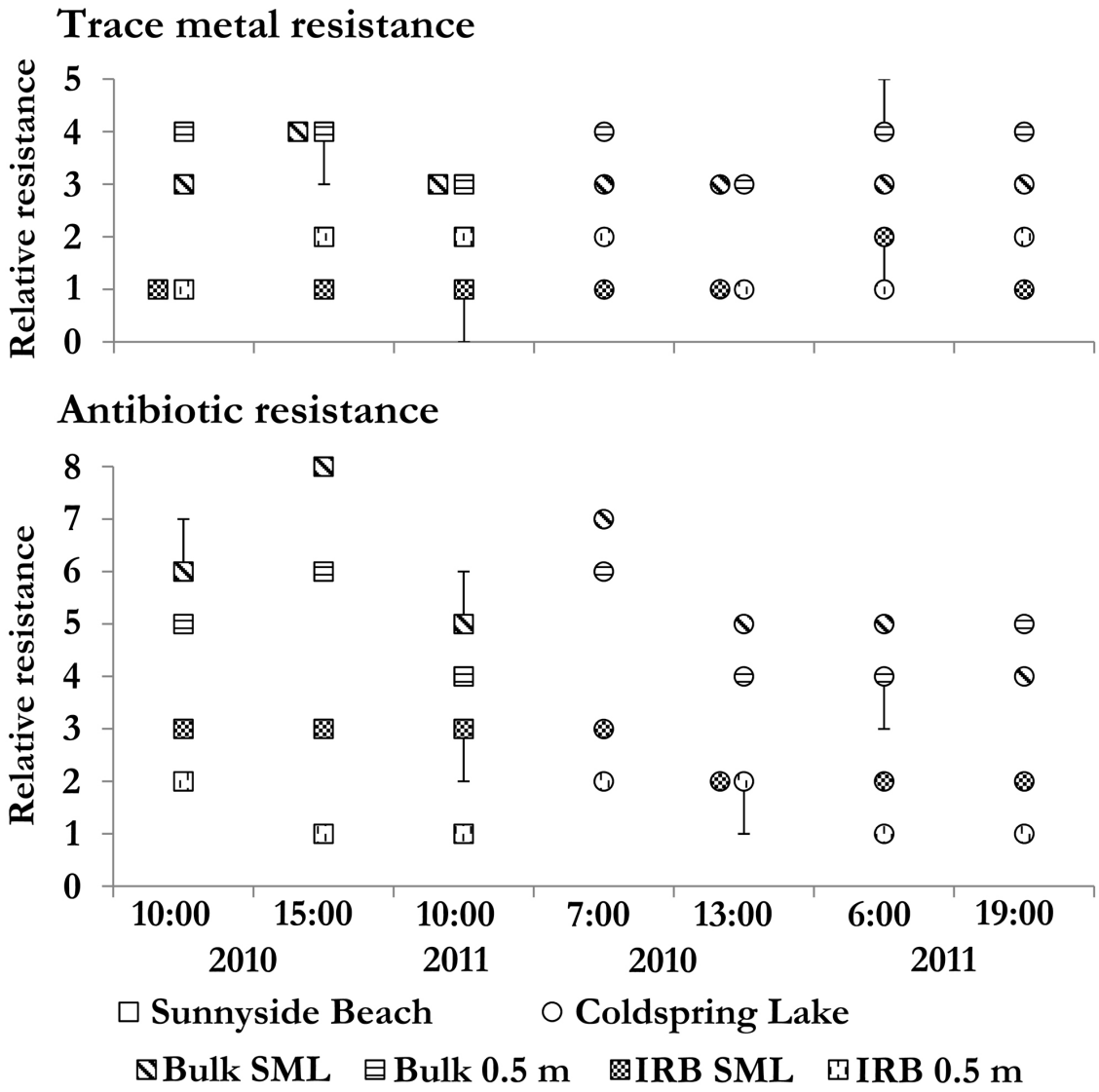

Figure 5.1 Resistance of soluble Fe(III)-reducing bacteria and other members of soluble Fe(III)-reducing enrichment communities from the SML and 0.5 m depth at Sunnyside Beach and Coldspring Lake to trace metal and antibiotic cocktails. Relative resistance is an ordinal scale based on the minimum cocktail concentrations at which wells were negative for visual indicators of Fe(III)-reducing bacteria (IRB) activity or bulk soluble Fe(III)-reducing enrichment community activity. Symbols represent the median (n=8) and error bars represent maximum and minimum values, when they differed from the median

Consistent with the soluble Fe(III)-reducing community resistance findings, the activity of solid Fe(III)-reducing bacteria in FeOOH-based microcosms inoculated with soluble Fe(III)-reducing enrichments from both sites and depths was inhibited by Cu exposure. Fe(II) concentrations in microcosms treated with 500 μM Cu acetate did not significantly ($p<0.05$) differ from sterile controls, indicating no significant reductive dissolution of FeOOH which would release Fe(II) into solution (Figure 5.2). However, Fe(II) concentrations were significantly ($p<0.05$) higher in untreated microcosms compared to those treated with Cu (Figure 5.2), identifying that bacteria capable of reducing solid Fe(III) were present. While Cu specifically inhibited the activity of solid Fe(III)-reducing bacteria in FeOOH-based microcosms, Cu exposure did not inhibit bulk community activity, which was consistently higher in Cu-treated microcosms compared to those not treated with Cu (Figure 5.2).

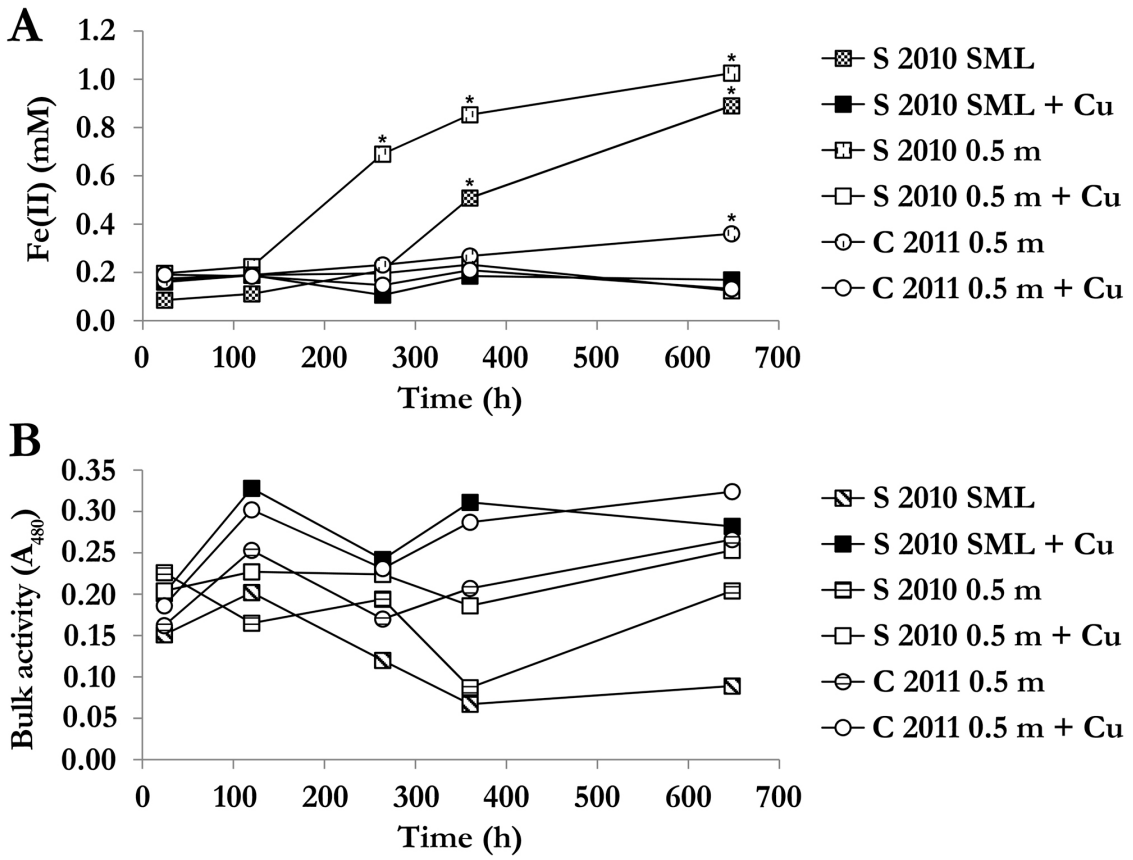

Figure 5.2 Effect of 500 μM Cu treatment on (a) Fe(II) production (i.e. activity of solid Fe(III)-reducing bacteria) and (b) bulk community activity in FeOOH-based microcosms inoculated with soluble Fe(III)-reducing communities enriched in the morning from the SML and 0.5 m depth at Sunnyside Beach (S) and Coldspring Lake (C). Data points represent the mean of triplicate microcosms. Standard errors did not exceed 0.05 mM for Fe(II) data points and 0.03 absorbance units for reduced tetrazolium absorbance data points. * Fe(II) concentration was significantly (p<0.05) lower in microcosms treated with 500 μM Cu compared to those left untreated

Exposure of soluble Fe(III)-reducing communities from both sites to Cu in FeOOH-based microcosms was also associated with the absence or reduced abundance of known Fe(III)-reducing genera in bacterial 16S rRNA gene clone libraries from Cu treated microcosms compared to those left untreated (Table 5.1). A member of the genus *Clostridium*,

which includes several Fe(III)-reducing species [8, 44, 45], constituted 39% of the clone library from Sunnyside Beach 2010 microcosms not treated with Cu, but was absent from the clone library from the corresponding Cu treated microcosms (Table 5.1). The clone library from microcosms treated with Cu (in which solid Fe(III) reducer activity was inhibited) was dominated (>75% of clones) by the genus *Sphingobacterium*, which made up only 20% of the library from untreated microcosms and does not include any known Fe(III) reducers (Table 5.1). Similarly, for the enrichment community recovered from 0.5 m depth at Coldspring Lake in 2011, a member of the genus *Aeromonas*, which includes a known Fe(III)-reducing bacterium (*A. hydrophila*) [46, 47] constituted only 17% of the clone library from Cu-treated microcosms but dominated (93%) the library from untreated microcosms (Table 5.1). The genus *Flavobacterium*, which does not include any known Fe(III)-reducing bacteria, dominated (62%) the clone library from Cu-treated microcosms but made up only 7% of the library from untreated microcosms containing Coldspring Lake 2011 enrichments from 0.5 m depth (Table 5.1).

Notably, 16S rRNA gene clone libraries derived from Fe(III)-reducing enrichment communities from both sites and depths included close sequence matches (99% sequence identity) to bacteria identified as causing disease in humans. These matches included strains of *Achromobacter xylosoxidans*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Elizabethkingia meningoseptica*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*.

Table 5.1 Effect of 500 μM Cu acetate treatment on the composition of bacterial 16S rRNA gene clone libraries from FeOOH-based microcosms inoculated with soluble Fe(III)-reducing communities enriched in the morning from the SML at Sunnyside Beach in 2010 (S 2010 SML) and at 0.5 m depth at Coldspring Lake in 2011 (C 2011 0.5 m)

Taxon	S 2010 SML	S 2010 SML + Cu	C 2011 0.5 m	C 2011 0.5 m + Cu
Alphaproteobacteria				
Ochrobactrum	+	+		
Betaproteobacteria				
Achromobacter	++	+		
Comamonas				
Gammaproteobacteria				
Aeromonas			++	+
Enterobacteriaceae				+
Pseudomonas				
Stenotrophomonas	+			
Firmicutes				
Clostridium	++			
Bacteroidetes				
Flavobacterium			+	++
Sphingobacterium	+	++		

+ = <25% of clone library, ++ = >25% of clone library

Across both sites and years, soluble Fe(III)-reducing communities enriched from the SML were significantly (p<0.05) more resistant to the cocktail of antibiotics compared to corresponding soluble Fe(III)-reducing enrichments from underlying waters (0.5 m depth) (Figure 5.1). More specifically, the soluble Fe(III)-reducing members of SML enrichment communities were also significantly (p<0.05) more resistant to the antibiotic cocktail compared to Fe(III)-reducing members of enrichments from 0.5 m depth (Figure 5.1). In contrast to these findings, based on their exposure to the trace metal cocktail, the overall

trace metal resistance of soluble Fe(III)-reducing enrichment communities and specifically their soluble Fe(III)-reducing members did not significantly (p<0.05) differ between enrichments from the SML compared to 0.5 m depth for both sites and years (Figure 5.1).

Based on their PCR-dependent detection, three mobile genetic elements known to carry genes encoding resistance to trace metals and antibiotics were present in soluble Fe(III)-reducing communities enriched from the SML and 0.5 m depth at both sites, but were more prevalent in enrichment communities from Sunnyside Beach (Table 5.2). Notably, IncP-1- and IncQ-like plasmids were detected exclusively in Sunnyside Beach SML enrichment communities (Table 5.2). Additionally, gel electrophoresis of gene cassette PCR products revealed the presence of distinct (i.e. differently sized) amplicons between the SML and 0.5 m depth soluble Fe(III)-reducing enrichment communities from both sites, pointing to depth-dependent community mobile genetic element diversity.

Table 5.2 PCR-detected (n=2) mobile genetic elements associated with trace metal and antibiotic resistance in soluble Fe(III)-reducing communities enriched in the morning at Sunnyside Beach (S) and Coldspring Lake (C) from the SML and 0.5 m depth

Element	**S 2010 SML**	**S 2010 0.5 m**	**S 2011 SML**	**S 2011 0.5 m**	**C 2010 SML**	**C 2010 0.5 m**	**C 2011 SML**	**C 2011 0.5 m**
Class 1 integron gene cassettes	+	+	++	++	+	+		
IncP-1-like plasmids			+					
IncQ-like plasmids	+		+					

For class 1 integron gene cassettes, + = one gel band (amplicon), ++ = multiple gel bands (amplicons)

5.5 Discussion

The observed lower resistance to trace metals and antibiotics among Fe(III)-reducing bacteria compared to other members of soluble Fe(III)-reducing enrichment communities does not support the selective development of resistance by Fe(III) reducers via their reductive dissolution of metal-laden Fe(III) phases. Rather, it indicates that trace metal or antibiotic exposure can selectively disrupt the Fe(III)-reducing capacity of aquatic bacterial communities to a greater extent compared to other metabolisms. This disruption would be expected to impact Fe cycling and associated biogeochemical processes (e.g. oxidative degradation of recalcitrant organic contaminants coupled to Fe(III) reduction by certain bacteria). Consistent with lower resistance among Fe(III) reducers within environmental bacterial communities, Markwiese and Colberg [48] observed that Cu exposure inhibited Fe(III) reduction by a sediment-derived enrichment community until dissolved Cu was almost completely removed from the aqueous phase by a resistant fermentative *Clostridium* sp.. Exposure to sulfonamide and tetracycline antibiotics inhibited bacterial Fe(III) reduction in soils at concentrations that did not affect general indicators of bacterial activity [49], indicating that antibiotic resistance is greater among other metabolic guilds compared to Fe(III) reducers.

In agreement with the observed inhibition of solid Fe(III) reducer activity in soluble Fe(III)-reducing enrichment communities treated with 500 μM Cu in this study, Fe(II) production was inhibited by 150 μM Cu in Fe(III)-reducing enrichments from metal contaminated creek bank soil [50] and by ~100 μM Cu in enrichments from metal contaminated river sediments [51]. The observation of greater bulk soluble Fe(III)-reducing enrichment community activity in microcosms treated with Cu compared to those left

untreated indicates that the introduction of Cu, as an acetate salt, promoted the activity of non-Fe(III)-reducing bacteria within these communities. Consistent with this explanation, Cu exposure has been demonstrated to stimulate the growth of environmental bacterial communities [2, 52]. Further, acetate is a relatively simple form of organic carbon that is readily utilized by many bacteria and so many have stimulated the activity of enrichment community members not otherwise inhibited by Cu exposure.

The reduced abundance of known Fe(III)-reducing genera in 16S rRNA gene clone libraries derived from FeOOH-based microcosms treated with Cu compared to untreated microcosms provided further evidence of the relatively low metal resistance of Fe(III) reducers within environmental bacterial communities. Contamination of soils with Cu has been shown to induce changes in the composition of resident bacterial communities such that certain taxonomic groups become dominant [3, 53]. Gram-negative bacteria dominated culturable isolates from metal contaminated soils and were specifically more tolerant to Cu compared to Gram-positive bacteria [54, 55]. In particular, *Flavobacterium* spp. constituted a major portion of isolates from soil contaminated with metal-rich sewage [56]. These literature findings are consistent with the observed selective decrease in the abundance of *Clostridium* spp. (Gram-positive) and increase in the abundance of *Flavobacterium* spp. (Gram-negative) in 16S rRNA gene clone libraries from the Sunnyside Beach enrichment community microcosms treated with Cu compared to those left untreated.

The observed similar magnitude of trace metal and antibiotic resistance in soluble Fe(III)-reducing communities enriched from an urban wastewater contaminated site and a remote pristine site indicates that mechanisms of chemical resistance are widespread in aquatic bacterial communities regardless of increased trace metal or antibiotic exposure via

anthropogenic contamination. Although this finding differs from previous observations of resistance to antibiotics and trace metals being higher in contaminated environments [14, 57], highly resistant bacteria have been found in remote pristine environments [58, 59].

Elevated antibiotic resistance and greater incidence of highly mobilizable broad-host-range mobile genetic elements in soluble Fe(III)-reducing enrichment communities from the SML compared to the underlying water column (0.5 m) point to the SML being a reservoir of antibiotic resistant bacteria within aquatic systems. These differences are in line with previous studies demonstrating higher levels of antibiotic resistance among bacteria inhabiting the SML compared to underlying waters [23-25]. The observation of higher resistance to antibiotics but not trace metals among soluble Fe(III)-reducing bacteria (and their larger parent enrichment communities) from the SML compared to 0.5 m depth is indicative of a lack of co-selection between metal and antibiotic resistance. Further, the lack of correspondence between antibiotic and metal resistance points to the development of antibiotic resistance via a mechanism independent of increased trace metal exposure, and subsequent co-selection of trace metal and antibiotic resistance, mediated by Fe(III) reduction. Elevated antibiotic resistance and the presence of distinct groups of mobile genetic elements in SML bacterial communities may stem from increased bacterial antagonism and associated antibiotic production in this habitat. Similar to the rhizosphere, an established environmental reservoir for bacteria possessing antagonistic traits such as antibiotic production and high resistance to multiple classes of antibiotics [60, 61], the freshwater SML is well suited to facilitating bacterial interactions within high density biofilms and flocs [17, 18].

Unlike Coldspring Lake, Sunnyside Beach was impacted by wastewater effluents, implicating wastewater-borne bacteria as the source of greater mobile genetic element diversity at the latter site. Supporting the increased prevalence of mobile genetic elements at Sunnyside Beach, the number and diversity of antibiotic resistance gene cassette types associated with class 1 integrons was previously found to be higher in flocs recovered at Sunnyside Beach compared to Coldspring Lake [62]. Further, IncP-1- and IncQ-like plasmids, which are among the few known plasmid groups that have broad host ranges, are highly mobilizable, and can carry genes encoding trace metal and antibiotic resistance [63, 64], were detected exclusively in Sunnyside Beach SML soluble Fe(III)-reducing enrichment communities. Their presence identifies the SML at this site as a possible site where antibiotic and trace metal resistance genes can be readily spread between bacteria. Supporting an important role for broad-host-range plasmids as a vehicle for resistance genes in the SML, IncP-1 plasmids carrying genes that encode resistance to several classes of antibiotics have been recovered from estuarine SML bacterial communities [65]. Further, air-liquid interfacial environments, such as the SML, are well suited to plasmid transfer [66]. In agreement with the detection of different plasmid types in soluble Fe(III)-reducing enrichment communities from the SML and 0.5 m depth, Dahlberg et al. [67] found little similarity between mercury resistance plasmids isolated from the SML and underlying waters of a marine site. This observation was attributed to substantial variation in bacterial community composition between depths [67]. Consistent with this explanation, depth-dependent differences in bacterial community composition have been observed in several freshwater settings including the sites investigated in this study [17, 68, 69]. Collectively, these results indicate that SML Fe(III)-reducing communities represent a distinct reservoir for mobile genetic resistance

determinants capable of disseminating resistance genes within diverse aquatic bacterial communities.

Supporting the observed potential presence of pathogenic bacteria in Fe(III)-reducing enrichment communities from the SML and 0.5 m depth, ubiquitous suspended flocs in these settings are well suited to harboring pathogens [70]. Flocs can protect pathogenic bacteria from environmental stressors including UV radiation that are relevant in surface waters [71]. The ability of *Pseudomonas aeruginosa*, detected in this study in enriched Fe(III)-reducing communities, to carry out extracellular electron transport has been proposed to contribute to its pathogenicity [72, 73], suggesting a degree of overlap between Fe(III) reducers and pathogens.

In summary, contrary to expectations, soluble Fe(III)-reducing bacteria were among the least metal or antibiotic resistant members of soluble Fe(III)-reducing enrichment communities. This finding suggests that other metabolic guilds also exhibit distinctive levels of metal and antibiotic resistance within environmental bacterial communities, which could substantially influence their functional contributions across variably contaminated environments. However, soluble Fe(III)-reducing enrichment communities demonstrated genetic and functional differences between the SML and 0.5 m depth with respect to traits including antibiotic resistance. These traits identify the SML as a potential distinct habitat for health-relevant bacteria.

5.6 Supplemental Material

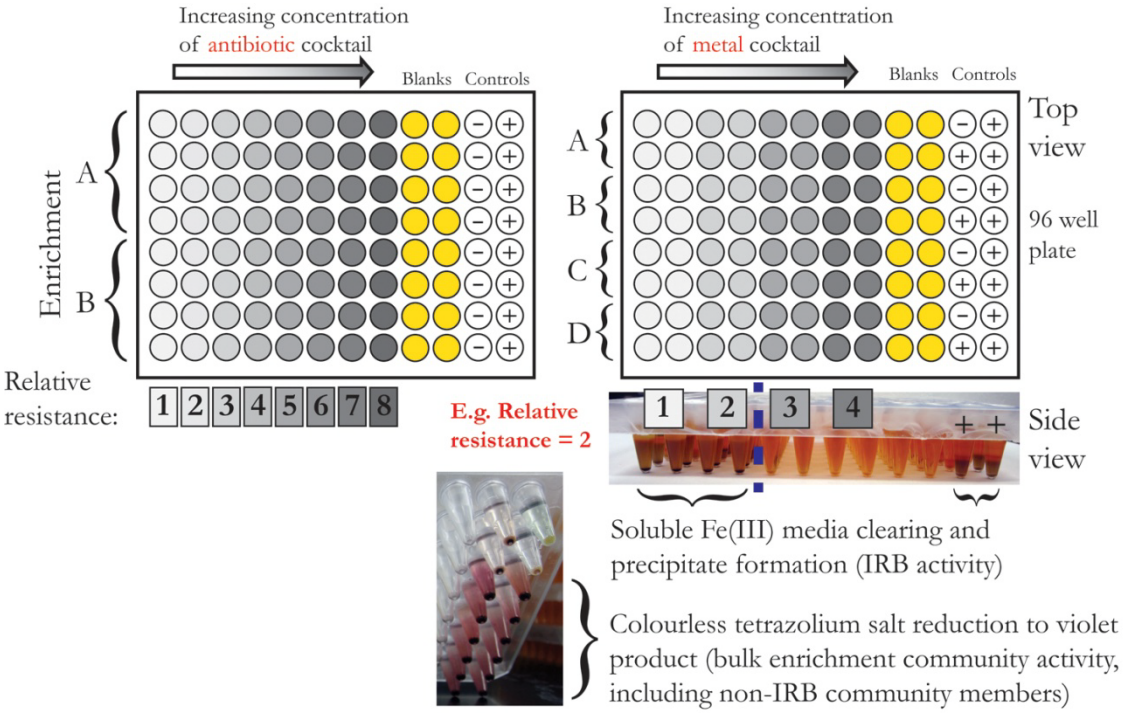

Figure S.5.1 A 96-well plate assay was employed to evaluate a range of concentrations of trace metal or antibiotic cocktails in order to establish the trace metal and antibiotic resistance of soluble Fe(III)-reducing bacteria (IRB) relative to other members of soluble Fe(III)-reducing enrichment communities.

5.7 References

1. Näslund J, Hedman JE, Agestrand C (2008) Effects of the antibiotic ciprofloxacin on the bacterial community structure and degradation of pyrene in marine sediment. Aquat Toxicol 90:223-227. doi: 10.1016/j.aquatox.2008.09.002
2. Magalhães CM, Machado A, Matos P, Bordalo AA (2011) Impact of copper on the diversity, abundance and transcription of nitrite and nitrous oxide reductase genes in

an urban European estuary. FEMS Microbiol Ecol 77:274-284. doi: 10.1111/j.1574-6941.2011.01107.x

3. Ranjard L, Echairi A, Nowak V, Lejon DP, Nouaïm R, Chaussod R (2006) Field and microcosm experiments to evaluate the effects of agricultural Cu treatment on the density and genetic structure of microbial communities in two different soils. FEMS Microbiol Ecol 58:303-315. doi: 10.1111/j.1574-6941.2006.00157.x
4. Córdova-Kreylos AL, Scow KM (2007) Effects of ciprofloxacin on salt marsh sediment microbial communities. ISME J 1:585-595. doi: 10.1038/ismej.2007.71
5. Tessier A, Rapin F, Carignan R (1985) Trace metals in oxic lake sediments: possible adsorption onto iron oxyhydroxides. Geochim Cosmochim Acta 49:183-194. doi: 10.1016/0016-7037(85)90203-0
6. El Bilali L, Rasmussen PE, Hall GEM, Fortin D (2002) Role of sediment composition in trace metal distribution in lake sediments. Appl Geochem 17:1171-1181. doi: 10.1016/s0883-2927(01)00132-9
7. Elliott AVC, Plach JM, Droppo IG, Warren LA (2012) Comparative floc-bed sediment trace element partitioning across variably contaminated aquatic ecosystems. Environ Sci Technol 46:209-216. doi: 10.1021/es202221u
8. Park HS, Kim BH, Kim HS, Kim HJ, Kim GT, Kim M, Chang IS, Park YK, Chang HI (2001) A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Clostridium butyricum* isolated from a microbial fuel cell. Anaerobe 7:297-306. doi: 10.1006/anae.2001.0399
9. Schröder I, Johnson E, de Vries S (2003) Microbial ferric iron reductases. FEMS Microbiol Rev 27:427-447. doi: 10.1016/s0168-6445(03)00043-3

10. Elliott AVC, Plach JM, Droppo IG, Warren LA (2014) Collaborative microbial Fe-redox cycling by pelagic floc bacteria across wide ranging oxygenated aquatic systems. Chem Geol 366:90-102. doi: 10.1016/j.chemgeo.2013.11.017
11. Lower SK, Hochella MF Jr, Beveridge TJ (2001) Bacterial recognition of mineral surfaces: nanoscale interactions between *Shewanella* and alpha-FeOOH. Science 292:1360-1363. doi: 10.1126/science.1059567
12. Gorby YA, Yanina S, McLean JS, Rosso KM, Moyles D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS, Culley DE, Reed SB, Romine MF, Saffarini DA, Hill EA, Shi L, Elias DA, Kennedy DW, Pinchuk G, Watanabe K, Ishii S, Logan B, Nealson KH, Fredrickson JK (2006) Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proc Natl Acad Sci USA 103:11358-11363. doi: 10.1073/pnas.0604517103
13. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006) Co-selection of antibiotic and metal resistance. Trends Microbiol 14:176-182. doi: 10.1016/j.tim.2006.02.006
14. Wright MS, Loeffler-Peltier G, Stepanauskas R, McArthur JV (2006) Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams. FEMS Microbiol Ecol 58:293-302. doi: 10.1111/j.1574-6941.2006.00154.x
15. Münster U, Heikkinen E, Knulst J (1997) Nutrient composition, microbial biomass and activity at the air-water interface of small boreal forest lakes. Hydrobiologia 363:261-270. doi: 10.1007/978-94-017-1493-8_21
16. Baastrup-Spohr L, Staehr PA (2009) Surface microlayers on temperate lowland lakes. Hydrobiologia 625:43-59. doi: 10.1007/s10750-008-9695-3

17. Drudge CN, Warren LA (2014) Diurnal floc generation from neuston biofilms in two contrasting freshwater lakes. Environ Sci Technol 48:10107-10115. doi: 10.1021/es503013w
18. Cunliffe M, Murrell JC (2009) The sea-surface microlayer is a gelatinous biofilm. ISME J 3:1001-1003. doi: 10.1038/ismej.2009.69
19. Grossart HP, Schlingloff A, Bernhard M, Simon M, Brinkhoff T (2004) Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. FEMS Microbiol Ecol 47:387-396. doi: 10.1016/s0168-6496(03)00305-2
20. Hakvåg S, Fjaervik E, Josefsen KD, Ian E, Ellingsen TE, Zotchev SB (2008) Characterization of *Streptomyces* spp. isolated from the sea surface microlayer in the Trondheim Fjord, Norway. Mar Drugs 6:620-635. doi: 10.3390/md6040620
21. Armstrong DE, Elzerman AW (1982) Trace metal accumulation in surface microlayers. J Great Lakes Res 8:282-287. doi: 10.1016/s0380-1330(82)71966-5
22. Wurl O, Obbard JP (2004) A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. Mar Pollut Bull 48:1016-1030. doi: 10.1016/j.marpolbul.2004.03.016
23. Hermansson M, Jones GW, Kjelleberg S (1987) Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. Appl Environ Microbiol 53:2338-2342.
24. Jones GW, Baines L, Genthner FJ (1991) Heterotrophic bacteria of the freshwater neuston and their ability to act as plasmid recipients under nutrient deprived conditions. Microb Ecol 22:15-25. doi: 10.1007/bf02540210

25. Azevedo JS, Araújo S, Oliveira CS, Correia A, Henriques I (2013) Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine environment. Microb Drug Resist 19:64-71. doi: 10.1089/mdr.2012.0084
26. Cunliffe M, Upstill-Goddard RC, Murrell JC (2011) Microbiology of aquatic surface microlayers. FEMS Microbiol Rev 35:233-246. doi: 10.1111/j.1574-6976.2010.00246.x
27. Kostka J, Nealson KH (1998) Isolation, cultivation and characterization of iron-and manganese-reducing bacteria. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G (eds) Techniques in microbial ecology. Oxford University Press, New York, pp 58-78
28. Loge FJ, Emerick RW, Thompson DE, Nelson DC, Darby JL (1999) Factors influencing ultraviolet disinfection performance Part I: Light penetration to wastewater particles. Water Environ Res 71:377-381. doi: 10.2175/106143097x122176
29. Hatzinger PB, Palmer P, Smith RL, Peñarrieta CT, Yoshinari T (2003) Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. J Microbiol Methods 52:47-58. doi: 10.1016/s0167-7012(02)00132-x
30. Tunney MM, Ramage G, Field TR, Moriarty TF, Storey DG (2004) Rapid colorimetric assay for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 48:1879-1881. doi: 10.1128/aac.48.5.1879-1881.2004

31. Silver S, Phung LT (2005) A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. J Ind Microbiol Biotechnol 32:587-605. doi: 10.1007/s10295-005-0019-6
32. Nriagu JO, Lawson G, Wong HKT, Cheam V (1995) Dissolved trace metals in lakes Superior, Erie, and Ontario. Envron Sci Technol 30:178-187. doi: 10.1021/es950221i
33. Zhang XX, Zhang T, Fang HH (2009) Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol 82:397-414. doi: 10.1007/s00253-008-1829-z
34. Kümmerer K (2009) Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75:417-434. doi: 10.1016/j.chemosphere.2008.11.086
35. Proia L, Lupini G, Osorio V, Pérez S, Barceló D, Schwartz T, Amalfitano S, Fazi S, Romaní AM, Sabater S (2013) Response of biofilm bacterial communities to antibiotic pollutants in a Mediterranean river. Chemosphere 92:1126-1135. doi: 10.1016/j.chemosphere.2013.01.063
36. Dong D, Li Y, Zhang J, Hua X (2003) Comparison of the adsorption of lead, cadmium, copper, zinc and barium to freshwater surface coatings. Chemosphere 51:369-373. doi: 10.1016/s0045-6535(02)00835-4
37. Boujelben N, Bouzid J, Elouear Z (2009) Adsorption of nickel and copper onto natural iron oxide-coated sand from aqueous solutions: study in single and binary systems. J Hazard Mater 163:376-382. doi: 10.1016/j.jhazmat.2008.06.128
38. Schwertmann U, Cornell RM (2000) Iron oxides in the laboratory. Wiley VHC, New York

39. Viollier E, Inglett PW, Hunter K, Roychoudhury AN, Van Cappellen P (2000) The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. Appl Geochem 15:785-790. doi: 10.1016/s0883-2927(99)00097-9
40. García-Balboa C, Vicente MS, Blázquez ML, González F, Muñoz JA, Ballester A (2011) Iron speciation in dissimilatory Fe(III)-reducing cultures. Geomicrobiology J 28:371-379. doi: 10.1080/01490451.2010.506100
41. Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F, Han SK (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol 28:141-155. doi: 10.3354/ame028141
42. Stokes HW, Holmes AJ, Nield BS, Holley MP, Nevalainen KM, Mabbutt BC, Gillings MR (2001) Gene cassette PCR: sequence-independent recovery of entire genes from environmental DNA. Appl Environ Microbiol 67:5240-5246. doi: 10.1128/aem.67.11.5240-5246.2001
43. Götz A, Pukall R, Smit E, Tietze E, Prager R, Tschäpe H, van Elsas JD, Smalla K (1996) Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Appl Environ Microbiol 62:2621-2628.
44. Dobbin PS, Carter JP, San Juan CG-S, von Hobe M, Powell AK, Richardson DJ (1999) Dissimilatory Fe(III) reduction by *Clostridium beijerinckii* isolated from freshwater sediment using Fe(III) maltol enrichment. FEMS Microbiol Lett 176:131-138. doi: 10.1016/s0378-1097(99)00229-3
45. Lehours AC, Batisson I, Guedon A, Mailhot G, Fonty G (2009) Diversity of culturable bacteria, from the anaerobic zone of the meromictic Lake Pavin, able to

perform dissimilatory-iron reduction in different *in vitro* conditions. Geomicrobiol J 26:212-223. doi: 10.1080/01490450902744012

46. Knight VV, Blakemore R (1998) Reduction of diverse electron acceptors by *Aeromonas hydrophila*. Arch Microbiol 169:239-248. doi: 10.1007/s002030050567
47. Pham CA, Jung SJ, Phung NT, Lee J, Chang IS, Kim BH, Yi H, Chun J (2003) A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Aeromonas hydrophila*, isolated from a microbial fuel cell. FEMS Microbiol Lett 223:129-134. doi: 10.1016/s0378-1097(03)00354-9
48. Markwiese JT, Colberg PJ (2000) Bacterial reduction of copper-contaminated ferric oxide: copper toxicity and the interaction between fermentative and iron-reducing bacteria. Arch Environ Contam Toxicol 38:139-146. doi: 10.1007/s002449910017
49. Thiele-Bruhn S, Beck IC (2005) Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. Chemosphere 59:457-465. doi: 10.1016/j.chemosphere.2005.01.023
50. Burkhardt EM, Bischoff S, Akob DM, Büchel G, Küsel K (2011) Heavy metal tolerance of Fe(III)-reducing microbial communities in contaminated creek bank soils. Appl Environ Microbiol 77:3132-3136. doi: 10.1128/aem.02085-10
51. Markwiese JT, Meyer JS, Colberg PJS (1998) Copper tolerance in iron-reducing bacteria: Implications for copper mobilization in sediments. Environ Toxicol Chem 17:675-678. doi: 10.1002/etc.5620170422
52. Guo X, Gu J, Gao H, Qin Q, Chen Z, Shao L, Chen L, Li H, Zhang W, Chen S, Liu J (2012) Effects of Cu on metabolisms and enzyme activities of microbial

communities in the process of composting. Bioresour Technol 108:140-148. doi: 10.1016/j.biortech.2011.12.087

53. Tom-Petersen A, Leser TD, Marsh TL, Nybroe O (2003) Effects of copper amendment on the bacterial community in agricultural soil analyzed by the T-RFLP technique. FEMS Microbiol Ecol 46:53-62. doi: 10.1016/s0168-6496(03)00192-2
54. Duxbury T, Bicknell B (1983) Metal-tolerant bacterial populations from natural and metal-polluted soils. Soil Biol Biochem 15:243-250. doi: 10.1016/0038-0717(83)90066-4
55. Kunito T, Nagaoka K, Tada N, Saeki K, Senoo K, Oyaizu H, Matsumoto S (1997) Characterization of Cu-resistant bacterial communities in Cu-contaminated soils. Soil Sci Plant Nutr 43:709-717. doi: 10.1080/00380768.1997.10414795
56. Barkay T, Tripp SC, Olson BH (1985) Effect of metal-rich sewage sludge application on the bacterial communities of grasslands. Appl Environ Microbiol 49:333-337.
57. Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, McArthur JV (2005) Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. Environ Sci Technol 39:3671-3678. doi: 10.1021/es048468f
58. Wani R, Kodam KM, Gawai KR, Dhakephalkar PK (2007) Chromate reduction by *Burkholderia cepacia* MCMB-821, isolated from the pristine habitat of alkaline crater lake. Appl Microbiol Biotechnol 75:627-632. doi: 10.1007/s00253-007-0862-7
59. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One 7:e34953. doi:10.1371/journal.pone.0034953

60. Berg G, Eberl L, Hartmann A (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environ Microbiol 7:1673-1685. doi: 10.1002/9781118297674.ch116
61. Mendes R, Garbeva P, Raaijmakers JM (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol Rev 37:634-663. doi: 10.1111/1574-6976.12028
62. Drudge CN, Elliott AVC, Plach JM, Ejim LJ, Wright GD, Droppo IG, Warren LA (2012) Diversity of integron- and culture-associated antibiotic resistance genes in freshwater floc. Appl Environ Microbiol 78:4367-4372. doi: 10.1128/aem.00405-12
63. Rawlings DE, Tietze E (2001) Comparative biology of IncQ and IncQ-like plasmids. Microbiol Mol Biol Rev 65:481-496. doi: 10.1128/mmbr.65.4.481-496.2001
64. Popowska M, Krawczyk-Balska A (2013) Broad-host-range IncP-1 plasmids and their resistance potential. Front Microbiol 4:44. doi: 10.3389/fmicb.2013.00044
65. Oliveira CS, Moura A, Henriques I, Brown CJ, Rogers LM, Top EM, Correia A (2013) Comparative genomics of IncP-1ε plasmids from water environments reveals diverse and unique accessory genetic elements. Plasmid 70:412-419. doi: 10.1016/j.plasmid.2013.06.002
66. Król JE, Nguyen HD, Rogers LM, Beyenal H, Krone SM, Top EM (2011) Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. Appl Environ Microbiol 77:5079-5088. doi: 10.1128/aem.00090-11
67. Dahlberg C, Linberg C, Torsvik VL, Hermansson M (1997) Conjugative plasmids isolated from bacteria in marine environments show various degrees of homology to

each other and are not closely related to well-characterized plasmids. Appl Environ Microbiol 63:4692-4697.

68. Hervas A, Casamayor EO (2009) High similarity between bacterioneuston and airborne bacterial community compositions in a high mountain lake area. FEMS Microbiol Ecol 67:219-228. doi: 10.1111/j.1574-6941.2008.00617.x
69. Hörtnagl P, Pérez MT, Zeder M, Sommaruga R (2010) The bacterial community composition of the surface microlayer in a high mountain lake. FEMS Microbiol Ecol 73:458-467. doi: 10.1111/j.1574-6941.2010.00904.x
70. Lyons MM, Ward JE, Gaff H, Hicks RE, Drake JM, Dobbs FC (2010) Theory of island biogeography on a microscopic scale: organic aggregates as islands for aquatic pathogens. Aquat Microb Ecol 60:1-13. doi: 10.3354/ame01417
71. Tang KW, Dziallas C, Grossart HP (2011) Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment. Environ Microbiol 13:378-390. doi: 10.1111/j.1462-2920.2010.02335.x
72. Zuo Y, Xing D, Regan JM, Logan BE (2008) Isolation of the exoelectrogenic bacterium *Ochrobactrum anthropi* YZ-1 by using a U-tube microbial fuel cell. Appl Environ Microbiol 74:3130-3137. doi: 10.1128/aem.02732-07
73. Wang Y, Wilks JC, Danhorn T, Ramos I, Croal L, Newman DK (2011) Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron acquisition. J Bacteriol 193:3606-3617. doi: 10.1128/jb.00396-11

CHAPTER 6: CONCLUSIONS

6.1 Summary

Results of this thesis reveal fundamental novel insight into the contaminant-relevant composition, physical structure, and function of microbial communities inhabiting the upper water column of littoral freshwater environments (Figure 6.1). Specifically, the SML was identified as a key site for the generation of trace metal sequestering and pathogen harbouring suspended flocs, enabled by the presence of a distinct biofilm-forming bacterioneuston community. Unlike the proximate underlying water column (0.5 m depth), flocs were generated in the SML from local biofilms in association with increased hydrodynamic forces that developed over diurnal timescales, identifying a potential mechanism by which atmosphere- and SML-sourced contaminants are transported to the underlying water column. Upper waters (SML and 0.5 m depth) were found to contain a phylogenetically diverse and metabolically flexible group of facultative Fe(III)-reducing bacteria that did not appreciably overlap with well-studied Fe(III)-reducing communities found in anoxic environments. Fe(III) reducers differed in their relative ability to reduce solid Fe oxyhydroxides, which are high capacity trace metal sorbents, between depths and systems due in part to depth- and system-dependent floc and soluble Fe(III) availability. These results identify biogeochemical characteristics affecting the ability of microbial communities to alter trace metal mobility in upper waters. Although Fe(III)-reducing bacteria were expected to be highly resistant to metals and antibiotics due to their ability to dissolve metal-rich Fe minerals, which would expose them to metals, Fe(III) reducers were relatively vulnerable to trace metal and antibiotic toxicity among bacteria enriched from upper waters.

Nevertheless, the observation of higher antibiotic resistance and a greater variety of mobile genetic elements in Fe(III)-reducing communities enriched from the SML relative to 0.5 m depth identified the former as a potential reservoir for microbial contaminants within freshwater lakes.

Collectively, the results of this thesis demonstrated the presence of a distinct SML community compared to the community inhabiting the proximate underlying water column (0.5 m depth), even though SML geochemistry largely reflected bulk characteristics of the water column. Specifically, the relative abundance of biofilm-associated phyla was greater in the SML community, detection of As-, Fe-, and S-metabolizing and/or obligately anaerobic bacteria was limited to the bacterioneuston, and enriched Fe(III)-reducing communities differed between depths with respect to their composition, relative capacity for solid Fe(III) reduction, and level of antibiotic resistance. Throughout this thesis, the composition, physical structure, and function of SML communities were demonstrated to be lake-specific, including with respect to how they differed from that in the proximate underlying water column (0.5 m depth). This lake specificity does not support the widespread presence of a consistently distinct bacterioneuston. Rather, this thesis identifies that the SML community differs from that observed in the underlying water column, but in a manner strongly influenced by the lake in which it is found. In particular, the lake-specific nature of the SML community and bacterial differences between the SML and proximate underlying water column were consistently linked to physical lake characteristics, with factors including energy regime magnitude (i.e. solar UV irradiance and wind speed) and its diurnal variability, water column depth, and degree of anthropogenic impact influencing the size and abundance of biofilms and flocs, the composition of Fe(III)-reducing bacterial populations, and the variety

of mobile genetic elements carried by bacterial communities. Notably, lake-specific bulk water column biogeochemistry (i.e. bacterial abundance, O_2 saturation, and concentrations of organic carbon, nutrients, and total Fe) was not found to appreciably influence the degree of floc generation and Fe(III) reduction in upper waters, emphasizing the importance of physical factors, as well as the intrinsic physical and chemical properties of the SML, as key controls on the composition, physical structure, and function of upper water column microbial communities.

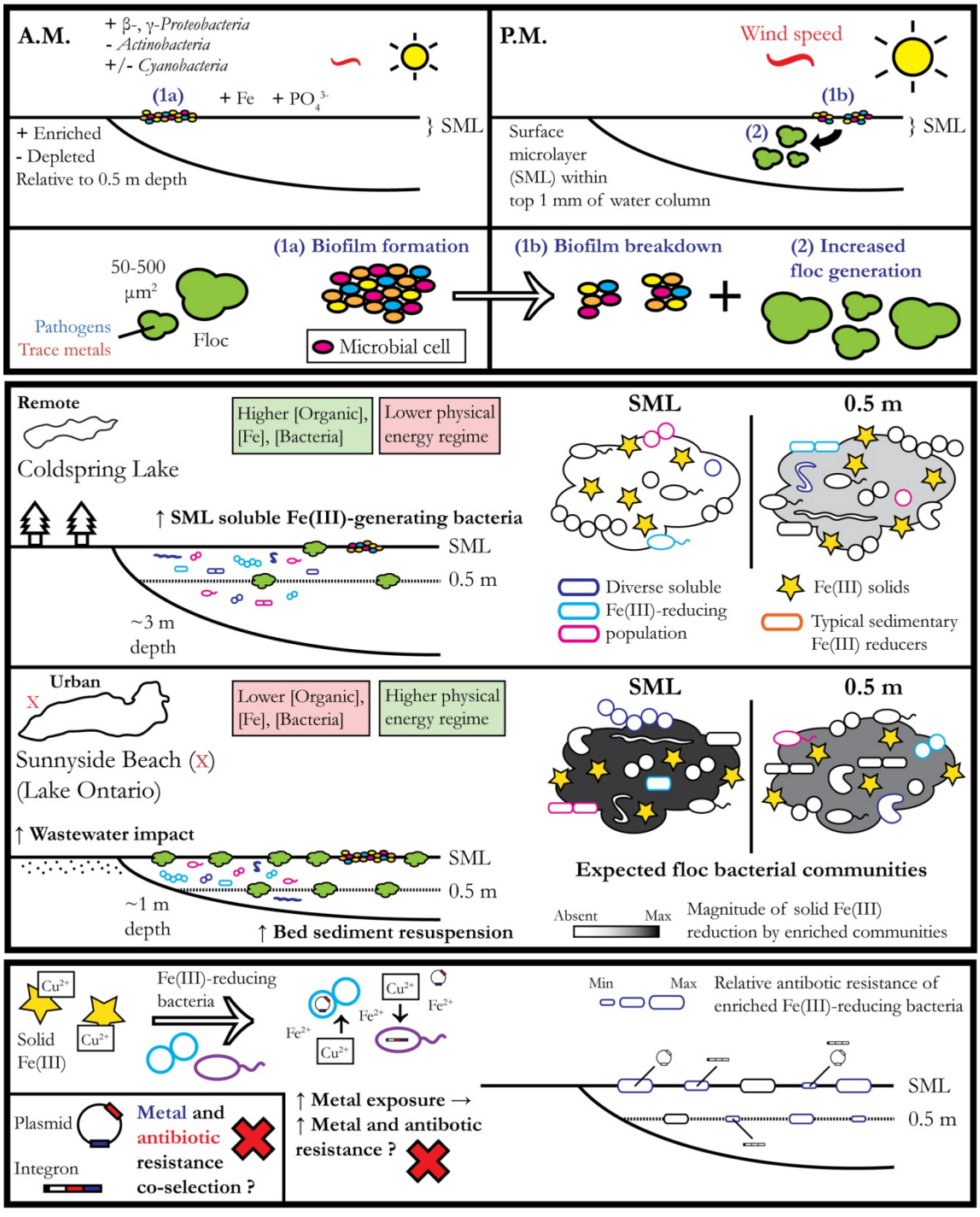

Figure 6.1 Summary of key thesis results. The upper water column of freshwater lakes was identified as an important site for microbial floc generation, Fe(III) reduction, and antibiotic resistance.

6.2 Knowledge Advancement

The results of this thesis advance our knowledge of the composition, physical structure, and function of the bacterioneuston community, a relatively poorly understood component of aquatic systems. They identify the SML as a biogeochemical hotspot in freshwater lakes with respect to the accumulation, transformation, and mobilization of major (C, Fe, P) and trace elements via floc generation and Fe(III) reduction, as well as a novel reservoir of antibiotic resistant bacteria capable of impacting human health. For the first time, the biogeochemical and health-relevant properties of the freshwater bacterioneuston community have been contextualized to the larger aquatic setting in which it is found, establishing how these properties are influenced by lake- and depth-specific physical, geochemical, and microbial characteristics. In addition, the well-oxygenated upper water column of freshwater lakes, including the SML, was found to contain a diverse and facultative population of soluble Fe(III)-reducing bacteria that while hypothesized to be highly chemically resistant due to its mobilization of trace metals from Fe(III) mineral phases, was consistently among the least metal or antibiotic resistant members of bulk enriched communities.

As the SML is a component of all surface waters and serves as their gateway to the atmosphere, the novel functions of its bacterial community identified in this thesis have the potential to substantially impact biogeochemical cycling and human health on a global scale. The generation of flocs from distinct SML biofilms represents an important, previously unknown mechanism by which microorganisms and other organic matter, nutrients, and trace metals, having accumulated in the SML, can be brought into contact with each other, thereby facilitating biogeochemical interactions and the development of health-relevant

characteristics, while providing a mechanism of transport (i.e. settling out of flocs from the SML) within aquatic systems. The presence of a distinct, diverse, and facultative Fe(III)-reducing bacterial population in well-oxygenated waters including the SML indicates it is the site of unexpectedly widespread transformation of Fe(III) derived from both the atmosphere and bulk water column not expected under bulk oxic conditions, impacting Fe bioavailability and the behaviour of Fe-associated nutrients and trace metal contaminants. Finally, the demonstration of greater antibiotic resistance among Fe(III)-reducing bacteria and their parent communities in the SML relative to the underlying water column highlights its potential role as a reservoir and point of dissemination of microbial contaminants.

Methods developed for this thesis represent an additional facet of its knowledge advancement. The need for a sampling device capable of rapidly collecting a larger volume of water (~2.5-3 L) from the SML, which is not possible with traditional SML samplers, was accomplished by the development of a SML sheet sampler from readily available materials. The collection of a larger water sample permitted the analysis of a greater suite of biogeochemical parameters than would have otherwise been possible. In addition, a 96-well plate assay was developed to assess the trace metal and antibiotic resistance of a specific metabolic guild, Fe(III)-reducing bacteria, relative to other members of larger parent enrichment communities. Instead of traditional methods based on measuring the inhibition of bacterial isolates by individual metals or antibiotics, this assay enabled a more environmentally-relevant approach whereby a subset of bacteria capable of a particular metabolic activity were compared to other members of environmental bacterial communities with respect to their inhibition by a cocktail of antibiotics or metals expected to be found in freshwater environments.

6.3 Future Research Directions

The capabilities of upper water column microbial communities and their implications for contaminant behaviour require further investigation, as outside of this thesis they remain poorly understood. In particular, the study of a large number of freshwater lakes and other freshwater environments (e.g. rivers and wetlands) would improve our understanding of how widespread the upper water column microbial characteristics (i.e. SML-based floc generation, Fe(III) reduction, and greater bacterioneuston antibiotic resistance) identified in this thesis are, as well as how they are influenced by variable physical, chemical, and microbial factors across different systems.

High throughput metagenomic DNA and RNA sequencing using next-generation methods could be employed to better distinguish the composition and functional characteristics of and differences between communities inhabiting the SML and proximate underlying water column, as well as to characterize the presence and expression of genes indicative of specific functions (e.g. floc generation, Fe(III) reduction, antibiotic resistance, horizontal gene transfer, and pathogenicity) identified in this thesis. This approach would clarify the distinctiveness of the bacterioneuston community and greatly expand our knowledge concerning the contaminant-relevant functional capabilities of microbial communities inhabiting the SML and proximate underlying waters. Complimenting a comprehensive investigation of upper water column microbial community genetic information, microscopic imaging based on the use of electrons or X-rays (i.e. environmental scanning electron microscopy equipped with energy dispersive X-ray spectroscopy, scanning transmission X-ray microscopy) could be employed to better understand how communities (i.e. biofilms and flocs) are physically structured. Images produced using these methods

would have substantially higher resolution than those reported in this thesis and in the currently published bacterioneuston literature, and could provide information regarding the spatial contextualization of bacteria with respect to elements that they are expected to interact with (e.g. Fe, P, trace metals). Further, fluorescent DNA and RNA probes could potentially be used in conjunction with microscopy to map out the locations and determine the abundance of specific bacteria such as Fe(III) reducers, highly antibiotic resistant bacteria, and pathogens in bacterioneuston communities, thereby adding to our knowledge of where and to what extent they operate in upper waters including the SML.

With respect to possible future research efforts concerning SML-based floc generation, a diel study involving hourly measurements of floc size and abundance, concurrent with the measurement of wind speed and solar irradiance, would permit a more comprehensive understanding of how floc generation is controlled by the physical energy regime of a lake. This approach could be used to investigate specific meteorological conditions (e.g. rainfall, high winds, long term quiescence) expected to influence floc generation, and more generally the composition of bacterioneuston communities. To clarify how the availability of floc (i.e. the abundance of flocs of different size) and soluble Fe(III) (i.e. concentrations of specific forms of organic-complexed Fe(III) including those derived from bacteria) influence the solid Fe(III)-reducing capacity of bacterial communities inhabiting the SML and proximate underlying water column, these three parameters could be measured concurrently across a large number of freshwater environments on multiple dates. This could be accompanied by the isolation of individual Fe(III)-reducing bacteria from both depths to specifically identify their capacity for reducing different forms of soluble and solid Fe(III) found in oxic freshwater environments, as well as determine the particular forms of

organic carbon they can utilize. These isolates could further be investigated with respect to their antibiotic resistance, mobile genetic element repertoire (i.e. clinically relevant antibiotic resistance genes), and pathogenicity to better understand their ability to impact human health. Specifically, a comprehensive investigation of upper water column microbial communities with respect to their inclusion of specific well known pathogenic bacteria (e.g. certain strains of *Escherichia coli*) bearing and expressing mobile antibiotic resistance genes, coupled with an assessment of the likelihood of human exposure to these bacteria (e.g. among swimmers at a public beach), could establish the degree of risk associated with these communities to human health. This information could be useful in the development of guidelines concerning recreational or occupational activities involving the upper waters of freshwater systems.

This thesis provides new knowledge regarding microbe-contaminant interactions in the upper water column of freshwater lakes, specifically identifying the presence of microbial communities capable of generating contaminant-sequestering flocs, reducing metal-sorbing Fe(III) minerals, and resisting the toxic effects of antibiotics. These functionalities are influenced by lake- and SML-specific physical and biogeochemical factors, indicating a nuanced regulation of contaminant presence by microbial communities in upper waters. This knowledge has important implications for assessing the ecological and human health impacts of contaminants in freshwater systems.

LIST OF REFERENCES

Abramoff MD, Magelhaes PJ, Ram SJ. 2004. Image processing with ImageJ. Biophotonics International 11(7):36-42.

Armstrong DE, Elzerman AW. 1982. Trace metal accumulation in surface microlayers. Journal of Great Lakes Research 8(2):282-287.

Azevedo JS, Araújo S, Oliveira CS, Correia A, Henriques I. 2013. Analysis of antibiotic resistance in bacteria isolated from the surface microlayer and underlying water of an estuarine environment. Microbial Drug Resistance 19(1):64-71.

Baastrup-Spohr L, Staehr PA. 2009. Surface microlayers on temperate lowland lakes. Hydrobiologia 625(1):43-59.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. 2006. Co-selection of antibiotic and metal resistance. Trends in Microbiology 14(4):176-182.

Balzano S, Statham PJ, Pancost RD, Lloyd JR. 2009. Role of microbial populations in the release of reduced iron to the water column from marine aggregates. Aquatic Microbial Ecology 54(3):291-303.

Boujelben N, Bouzid J, Elouear Z. 2009. Adsorption of nickel and copper onto natural iron oxide-coated sand from aqueous solutions: study in single and binary systems. Journal of Hazardous Materials 163(1):376-382.

Boyd PW, Mackie DS, Hunter KA. 2010. Aerosol iron deposition to the surface ocean — Modes of iron supply and biological responses. Marine Chemistry 120(1-4):128-134.

City of Toronto. 2014a. Humber Wastewater Treatment Plant 2013 Annual Report. http://www1.toronto.ca/City%20Of%20Toronto/Toronto%20Water/Files/pdf/H/Humber%20TP%20Annual%20Report%202013.pdf

City of Toronto. 2014b. Toronto Beaches Water Quality. http://app.toronto.ca/tpha/beaches.html

Crawford RL, Johnson L, Martinson M. 1982. Bacterial enrichments in surface films of freshwater lakes. Journal of Great Lakes Research 8(2):323-325.

Cunliffe M, Upstill-Goddard RC, Murrell JC. 2011. Microbiology of aquatic surface microlayers. FEMS Microbiology Reviews 35(2):233-246.

Dong D, Li Y, Zhang J, Hua X. 2003. Comparison of the adsorption of lead, cadmium, copper, zinc and barium to freshwater surface coatings. Chemosphere 51(5):369-373.

Droppo IG. 2001. Rethinking what constitutes suspended sediment. Hydrological Processes 15(9):1551-1564.

Dutka BJ, Kwan KK. 1978. Health-indicator bacteria in water-surface microlayers. Canadian Journal of Microbiology 24(2):187-188.

Edge TA, Hill S, Seto P, Marsalek J. 2010. Library-dependent and library-independent microbial source tracking to identify spatial variation in faecal contamination sources along a Lake Ontario beach (Ontario, Canada). Water Science and Technology 62(3):719-727.

Elliott AVC, Plach JM, Droppo IG, Warren LA. 2012. Comparative floc-bed sediment trace element partitioning across variably contaminated aquatic ecosystems. Environmental Science and Technology 46(1):209-216.

Elliott AVC, Plach JM, Droppo IG, Warren LA. 2014. Collaborative microbial Fe-redox cycling by pelagic floc bacteria across wide ranging oxygenated aquatic systems. Chemical Geology 366:90-102.

Environment Canada. 1979. Analytical methods manual. Inland Waters Directorate, Water Quality Branch: Ottawa, ON.

Environment Canada. 2011. National Climate Data and Information Archive. http://climate.weather.gc.ca/

Gorby YA, Yanina S, McLean JS, Rosso KM, Moyles D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS, Culley DE, Reed SB, Romine MF, Saffarini DA, Hill EA, Shi L, Elias DA, Kennedy DW, Pinchuk G, Watanabe K, Ishii S, Logan B, Nealson KH, Fredrickson JK. 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proceedings of the National Academy of Sciences 103(30):11358-11363.

Götz A, Pukall R, Smit E, Tietze E, Prager R, Tschäpe H, van Elsas JD, Smalla K. 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Applied and Environmental Microbiology 62(7):2621-2628.

Grossart HP, Schlingloff A, Bernhard M, Simon M, Brinkhoff T. 2004. Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. FEMS Microbiology Ecology 47(3):387-396.

Hakvåg S, Fjaervik E, Josefsen KD, Ian E, Ellingsen TE, Zotchev SB. 2008. Characterization of *Streptomyces* spp. isolated from the sea surface microlayer in the Trondheim Fjord, Norway. Marine Drugs 6(4):620-635.

Hatzinger PB, Palmer P, Smith RL, Peñarrieta CT, Yoshinari T. 2003. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. Journal of Microbiological Methods 52(1):47-58.

Henk MC. 2004. Method for collecting air-water interface microbes suitable for subsequent microscopy and molecular analysis in both research and teaching laboratories. Applied and Environmental Microbiology 70(4):2486-2493.

Hermansson M, Jones GW, Kjelleberg S. 1987. Frequency of antibiotic and heavy metal resistance, pigmentation, and plasmids in bacteria of the marine air-water interface. Applied and Environmental Microbiology 53(10):2338-2342.

Jones GW, Baines L, Genthner FJ. 1991. Heterotrophic bacteria of the freshwater neuston and their ability to act as plasmid recipients under nutrient deprived conditions. Microbial Ecology 22(1):15-25.

Kostka J, Nealson KH. 1998. Isolation, cultivation and characterization of iron-and manganese-reducing bacteria. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G, editors. Techniques in Microbial Ecology. New York, NY: Oxford University Press. P 58-78.

Lovley DR. 2013. Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes. New York, NY: Springer. P 287-308.

Lower SK, Hochella MF Jr, Beveridge TJ. 2001. Bacterial recognition of mineral surfaces: nanoscale interactions between *Shewanella* and alpha-FeOOH. Science 292(5520):1360-1363.

Lu S, Chourey K, Reiche M, Nietzsche S, Shah MB, Neu TR, Hettich RL, Küsel K. 2013. Insights into the structure and metabolic function of microbes that shape pelagic iron-rich aggregates ("iron snow"). Applied and Environmental Microbiology 79(14):4272-4281.

Lyons MM, Ward JE, Gaff H, Hicks RE, Drake JM, Dobbs FC. 2010. Theory of island biogeography on a microscopic scale: organic aggregates as islands for aquatic pathogens. Aquatic Microbial Ecology 60:1-13.

Mahowald NM, Engelstaedter S, Luo C, Sealy A, Artaxo P, Benitez-Nelson C, Bonnet S, Chen Y, Chuang PY, Cohen DD, Dulac F, Herut B, Johansen AM, Kubilay N, Losno R, Maenhaut W, Paytan A, Prospero JM, Shank LM, Siefert RL. 2009. Atmospheric iron deposition: global distribution, variability, and human perturbations. Annual Review of Marine Science 1:245-278.

Maki JS, Hermansson M. 1994. The dynamics of surface microlayers in aquatic environments. In: Wotton RS, editor. The Biology of Particles in Aquatic Systems. Boca Raton, FL: Lewis. P 161-182.

Molin S, Tolker-Nielsen T. 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Current Opinion in Biotechnology 14(3):255-261.

Moore TR. 1985. The spectrophotometric determination of dissolved organic carbon in peat waters. Soil Science Society of America Journal 49(6):1590-1592.

Münster U, Heikkinen E, Knulst J. 1997. Nutrient composition, microbial biomass and activity at the air-water interface of small boreal forest lakes. Hydrobiologia 363(1-3):261-270.

Nazzal Y, Rosen MA, Al-Rawabdeh AM. 2013. Assessment of metal pollution in urban road dusts from selected highways of the Greater Toronto Area in Canada. Environmental Monitoring and Assessment 185(2):1847-1858.

Ontario Ministry of Natural Resources. 1998. Algonquin Provincial Park Management Plan. http://www.algonquinpark.on.ca/pdf/management_plan.pdf

Paerl HW, Pinckney JL. 1996. A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. Microbial Ecology 31(3):225-247.

Plach JM, Elliott AV, Droppo IG, Warren LA. 2011. Physical and ecological controls on freshwater floc trace metal dynamics. Environmental Science and Technology 45(6):2157-2164.

Plusquellec A, Beucher M, Le Lay C, Le Gal Y, Cleret JJ. 1991. Quantitative and qualitative bacteriology of the marine water surface microlayer in a sewage-polluted area. Marine Environmental Research 31(3):227-239.

Santos L, Santos AL, Coelho FJ, Gomes NC, Dias JM, Cunha Â, Almeida A. 2011. Relation between bacterial activity in the surface microlayer and estuarine hydrodynamics. FEMS Microbiology Ecology 77(3):636-646.

Simon M, Grossart HP, Schweitzer B, Ploug H. 2002. Microbial ecology of organic aggregates in aquatic ecosystems. Aquatic Microbial Ecology 28(2): 175-211.

Södergren A. 1993. Role of aquatic surface microlayer in the dynamics of nutrients and organic compounds in lakes, with implications for their ecotones. Hydrobiologia 251(1-3):217-225.

Stokes HW, Holmes AJ, Nield BS, Holley MP, Nevalainen KM, Mabbutt BC, Gillings MR. 2001. Gene cassette PCR: sequence-independent recovery of entire genes from environmental DNA. Applied and Environmental Microbiology 67(11):5240-5246.

Stolle C, Nagel K, Labrenz M, Jürgens K. 2010. Succession of the sea-surface microlayer in the coastal Baltic Sea under natural and experimentally induced low-wind conditions. Biogeosciences 7:2975-2988.

Stolle C, Labrenz M, Meeske C, Jürgens K. 2011. Bacterioneuston community structure in the southern Baltic sea and its dependence on meteorological conditions. Applied and Environmental Microbiology 77(11):3726-3733.

Tang KW, Dziallas C, Grossart HP. 2011. Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment. Environmental Microbiology 13(2):378-390.

Viollier E, Inglett PW, Hunter K, Roychoudhury AN, Van Cappellen P. 2000. The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. Applied Geochemistry 15(6):785-790.

Wotton RS, Preston TM. 2005. Surface films: Areas of water bodies that are often overlooked. BioScience 55(2):137-145.

Wurl O, Obbard JP. 2004. A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. Marine Pollution Bulletin 48(11-12):1016-1030.

Zachara JM, Fredrickson JK, Smith SC, Gassman PL. 2001. Solubilization of Fe (III) oxide-bound trace metals by a dissimilatory Fe (III) reducing bacterium. Geochimica et Cosmochimica Acta 65(1):75-93.

Zimmermann-Timm H. 2002. Characteristics, dynamics and importance of aggregates in rivers – an invited review. International Review of Hydrobiology 87(2-3):197-240.