

**The Ecology, Physiology, and Diversity of Aerobic Anoxygenic Phototrophs in Lake Winnipeg,
Canada.**

by

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Abstract

Aerobic anoxygenic phototrophs (AAP) are a group of photoheterotrophic bacteria that use sunlight as a supplemental energy source, allowing them to be found in many illuminated environments. Here, we investigated their presence in Lake Winnipeg, Manitoba, Canada with spatial and temporal considerations to define the niche that they fill, and understand the biogeochemical pathways influenced or controlled by AAP. In addition, we explored their diversity and expanded upon the range of photosynthetic arrangements known, by looking into a variety of newly purified pigmented isolates to assess unique characteristics, all of which allowed us to better understand the AAP group as a whole.

We described seasonal trends of microbial communities in Lake Winnipeg, where up to 1.49% of the sequenced community and 19.1% of the total culturable copiotrophic heterotrophs were AAP at littoral zones during their peak spring season, and they directly followed sunlight availability. Isolated and purified strains were capable of several metal transformations, including metal(loid) oxide reduction and production of siderophores or metallophores. This widespread physiology, particularly among lake isolates, suggested a potential ecological importance for AAP involving a metal(loid) collecting niche. AAP with elongated cells were established as both associated to, and possibly preyed upon by filter feeding mollusks *Dreissena polymorpha*, which provided insights into how these bacteria interact with the foodweb. Many pigmented and phylogenetically diverse strains were discovered from across the lakes euphotic zones. Representatives of each different species were physiologically, morphologically and phylogenetically compared and described, showing members of specific taxonomic families all shared heritable traits including pigments. This confirmed evolutionary age of photosynthesis in AAP, and suggested recent horizontal gene transfer events did not occur. Furthermore, unusual aerobic violet pigmented strains that came from under the frozen surface during winter were explored. Overall, the study of Lake Winnipeg microbial communities led to several important discoveries: Seasonal dynamics found that AAP followed daylight trends rather than any other environmental parameter; These phototrophs were likely preyed upon by filter feeding mollusks; Several new metal transformation capabilities were described; Unique pigmentation was found. All combined considerations confirmed the importance of AAP as regular members of this freshwater ecosystem, with high numbers and large diversity, likely contributing significantly to the foodweb.

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List of Abbreviations

AAP	aerobic anoxygenic phototroph
AnAnP	anaerobic anoxygenic phototroph
AOP	aerobic oxygenic phototroph
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
BG-11	AOP medium
BLAST	Basic Local Alignment Search Tool
BLM	blue lake medium
BPheo	bacteriopheophytin
BSCA	biological soil crust medium A
BSCB	biological soil crust medium B
CAS	chromeazurool S
CFU	colony forming unit
Chl	chlorophyll
CRC ICPMS	collision/reaction cell-based inductively coupled plasma mass spectrometry
Crt	carotenoids
Cyt	cytochrome
ddH ₂ O	distilled deionized water
DAPI	4',6-diamidino-2-phenylindole
DCM	dichloromethane
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
e-	electron
eDNA	environmentally sourced DNA
ESDIS	Earth Science Data and Information System
ETC	electron transport chain
GC	gas chromatography
GETS	Graduate Enhancement of Tri-Council Stipends
HDTMA	hexadecyltrimethylammonium
HGT	horizontal gene transfer
HPLC	high pressure liquid chromatography
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
ICP	inductively coupled plasma
LANCE	Land Atmosphere Near-real time Capability for EOS
LC	liquid chromatography

LDAO	lauryldimethylamine N-oxide
LH	light harvesting
LWRC	Lake Winnipeg Research Consortium
Lux	light intensity
MA	medium A
MAG	metagenome assembled genome
MB	medium B
MCL	Maximum Composite Likelihood
MALDI	matrix-assisted laser desorption/ionization
MIC	minimum inhibitory concentration
MS	mass spectrometry
M.V.	motor research vessel
N ₁	medium N ₁ of Mahoney Lake strains
NADH	nicotinamide adenine dinucleotide
NASA	National Aeronautics and Space Administration
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance spectroscopy
NSERC	National Science and Engineering Research Council
OD	optical density
OM	oligotrophic medium
OUT	operational “taxonomic” unit
P+	excited bacteriochlorophyll
PAGE	polyacrylamide gel electrophoresis
PCoA	principle coordinate analysis
PGC	photosynthetic gene cluster
Pheo	pheophytin
PM	potato based medium
PNSB	purple non-sulfur bacteria
PNSM	PNSB medium
ppm	parts per million
PS	photosystem
Q	quinone
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
RC	reaction centre
RO	rich organic medium

RO _T	RO with 100µg/mL tellurite
RuBisCO	ribulose biphosphate carboxylase/oxygenase
ROS	radical oxygen species
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography
USA	United States of America

1. Chapter 1.
Introduction

1.1. Water Ecosystem Organization, Troubles, and Importance

1.1.1. About water chemistry and significance

All things considered, it would appear that liquid water, H_2O , is a fundamental component of life (Brack, 1993; Rothschild and Mancinelli, 2001). Absolutely required at an aqueous state as a solvent that allows passive diffusion of materials, supporting chemical reactions between carbon and other atoms arranged in complex molecules that assemble every biological form on the planet Earth (Miller and Urey, 1959). Without H_2O , gradients of materials could not be created, reactions would occur too infrequently with fluid equilibriums unable to establish, hindering life as we know it.

Therefore, understanding locations with any volume of this liquid is important for comprehending carbon-based life. On Earth, water makes up to 71% of its surface. Most is saline at about 3.5% NaCl and makes up the world's great oceans, with many organisms acclimated to this solute. In comparison, a small proportion of the available H_2O (about 2.5%) has less osmotic pressure and contains <0.35% NaCl, commonly known as freshwater (Gleick, 1993). Of that, two thirds are cold enough to be in solid-phases of ice and snow, considered too harsh and unable to support life (if the ice and snow are indeed frozen, a feature discussed later in this work).

Remarkably, numerous creatures depend on the remaining one-third, or 0.76% of total water on the planet to thrive. This includes a majority of land plants, animals, and entire terrestrial ecosystems that all require freshwater to grow, survive, and reproduce. This is to say, that while modern speculations suggest that life originated in oceans ~3.8 Ga in the past (Mojzsis *et al.*, 1996), the conquest of land happened comparatively recently, ~0.5 Ga ago. Yet, many complex and remarkable varieties of organisms that have existed among terrestrial habitats rely on freshwater. Considered to be a finite and important resource, it is crucial to understand and protect the most accessible reservoirs.

In this way, lakes are the named largest accumulations of freshwater that make up a mere 0.013% of all H_2O . While many thousands of small lakes and ponds exist on the planet, the biggest basins have been shown to host equally immense ecosystems. These permanent fixtures and habitat forming areas are separated and different enough in both physical and chemical characteristics to have allowed for evolution to occur specifically to each location.

1.1.3. Aquatic food webs

While every freshwater habitat is shaped by its chemical and physical traits that can be classically measured, our modern understanding of lake ecology has come from both historical analysis, and recent findings that reshaped conventional dogmas. Traditional “food chain” mentality of simplified predation profiles has been replaced with “foodwebs” that more appropriately take into account several different aspects. It is now understood that the fluctuation of one parameter in a water system (physical, chemical or biological, such as temperature, pH or a population size, respectively) can affect the ecosystem in multiple ways (Tanentzap *et al.*, 2020), rather than only one simple part.

But in general, lake ecosystems are quite stable due to their size, and have consistent biological members. Most freshwater lakes contain large piscivorous fish that consume planktivorous fish that devour zooplankton, which in turn digest microorganisms and soluble nutrients (Crowder *et al.*, 1988). Mammals hunt various fish species, while mollusks are believed to feed on zooplankton and smaller organisms or organics (Hwang *et al.*, 2004). The assortment and relative amount of each population within a community are dependent on the habitat conditions. Predation profiles have been established around both season and the maturation of offspring, in some cases being different from larvae to adult. However, while a majority of the mammals and fish have already been well identified, observed, and dissected due to their large size and ease of surveillance (Madenjian *et al.*, 2002), several of the smaller creatures remain unknown, with continual discoveries yearly. Such is the case with microscopic organisms, that are incredibly diverse yet laborious to study, as discussed later.

Further, while many individual species have been tracked, modern science has been devoted to entire system dynamics to fully appreciate why certain populations fluctuate (Tanentzap *et al.*, 2020). The oscillations of one creature are linked in connection to many others, or to ecosystem chemistry and physicality. One such example is the emerald shiner, *Notropis atherinoides*, which are abundant in most North American lakes. They consume copepodids, daphnids or *Keratella* spp., but are preyed upon by walleye, *Sander vitreus*, therefore will increase or decrease in population, respectively, if either of those two populations grow (Campbell and MacCrimmon, 1970). Moreover, minor increases in algae benefit the minnows, but too much microbial turbidity can have a negative effect (Nieman and Gray, 2020). By understanding each association, scientists hope to determine key features that cause either the rise or decline of particular organisms, in most cases to prevent extinction. As more lake habitats are analyzed, each is

slowly being characterized with unique ecological systems. It is clear many biomes are quite important as reservoirs of biological diversity, potentially having undiscovered value that could provide insight into understanding life and growth capabilities not found elsewhere (Timoshkin, 2001).

As mankind has spread across the planet, these ecosystems began to take on additional importance. Sources of fisheries, drinking water, irrigation, and other means of human activity became facets of great responsibility, as water reservoirs could be unfortunately spoiled if not handled and used with care.

1.1.4. The unfortunate issues affecting lakes

Many negative influences by people are often done not in ill wishes, but rather due to self-preservation and ignorance of negligent repercussions. Regrettably, this has occurred within much of our terran habitat. Human byproducts have been repetitively discarded in the easiest, but not ecologically safest fashion, without the realization of how such refuse changes or damages local environments, particularly if provided in vast amounts. Industrial manufacturing and disposal (Greig *et al.*, 2010), energy production and usage (Algera *et al.*, 2020), or the pumping of wastes into waterways (Schindler *et al.*, 2012), are just some of the negative anthropogenic sources of habitat modification. Of course human near-sightedness does not end here, but also includes unreasonable hunting that incurred extinction of many species, terraforming of areas with little regard to sensitive ecosystems, and severe gaseous pollution that is likely modifying our planet with consequences scientists can only speculate (Vasseur *et al.*, 2014). Predictions suggest that the changes we are promoting are probably too harmful and haphazard to continue, particularly when we do not know the extent of potential negative repercussions (Hoegh-Guldberg and Bruno, 2010).

Without realizing, several important freshwater habitats and lakes have been affected and now suffer from such unforeseen downstream consequences (Tellier *et al.*, 2022). The first of which is where such a phrase gets its meaning: Pollution, or rather the act of dumping diluted waste into waterways, makes it no longer the problem of the person upstream, but that of where it flows to. In this way, eutrophication and toxification of aquatic habitats have occurred after prolonged supply of excess organic nutrients or chemicals that were added straight to waterways or within their drainage basins. Even when not directly added to streams, rivers, ponds, lakes, or oceans, materials from land are easily transferred into aquatic systems from natural forecasts of wind and rain (Müller *et al.*, 2020). Eutrophication can be caused by runoff of fields laden with fertilizers, intended to make plants grow better, as well as by human waste water (Stauffer, 2013). While this includes the effluent from sewage treatment plants, it also takes into account the direct discharge

from industry accumulation ponds. One dramatic example is of lumber mills in the drainage basin of Lake Baikal that expel concentrated organics into their rivers that flow into the largest freshwater lake in the world (Lubomudrov, 1978). While once considered extremely oligotrophic, or of minimal organic content, it is slowly becoming polluted with organics.

1.1.5. Eutrophication consequences

The addition of nutrients to a habitat has been linked to drastically changed ecosystem dynamics, including increased turbidity of water, changes in certain species relative abundance, and in worst cases, the extinction of fish populations and toxicity of water for human consumption. Many studies have analyzed such abrupt alterations, particularly in the Great Lakes of the Laurentian Bay (Meyers, 2006). Much like how land vegetation was intended to be stimulated by addition of fertilizers, the external introduction of phosphorus and nitrogen to waters has caused large blooms of aquatic 'plants', including microscopic algae and cyanobacteria, as such compounds are typically their growth-limiting factors in oligotrophic environments (Dolman *et al.*, 2012). The microbial flourish has been shown to disrupt natural ecosystem balance, and in some cases their biomass got so dense, that the lower lake zone became anoxic, where fish populations suffocated and diminished (Havens, 2008). However, as discussed above in regard to food webs, algae and fish are not the only organisms in the natural ecosystem. Zooplankton, mollusks, and other animals can all be affected by this stark change in conditions. Also, the diverse microorganisms present are likely to be specifically afflicted as nutrient availability adjusts. In this way, excess nutrients can lead to the decline in valuable fish numbers, transformations in desirable ecosystem diversity, and potential poisoning if biological toxins are produced, making the water undrinkable (Miller *et al.*, 2017).

1.1.6. Metals and other chemical compounds

In comparison, the accumulation of chemicals, including metals, small organic molecules, salts and other solutes can also modify the environment. Such unfortunate effluent has included mine tailings, which emit the unwanted compounds in washes during the collection and purification steps of desirable minerals (Hughes *et al.*, 2017). Many metals and their oxides are toxic to many forms of life, even at low concentrations, such as 1 ppm for tellurite (Maltman *et al.*, 2015). When present in moderate amounts in the water column, an unfortunate result of food web predation profiles is the bioaccumulation of metals in the tissues of some higher-level organisms, which has been associated to increased mortality rates (Bryan and Darracott, 1979; Luoma and Rainbow, 2005). This is why care is advised for human consumption of shellfish such as oysters for fear of high metal content including mercury (Mok *et al.*, 2015).

If metals or metal(loid) oxides are found at staggeringly higher concentrations in water, then their toxicity effectively inhibits most forms of higher life, making such areas practically devoid of vegetation, fish, and nearly all other eukaryotes. The only biology that remains, or establishes in such areas are the sole members capable of extreme tolerance, microorganisms (Hughes *et al.*, 2017). This has been found in some waterbodies, causing issues similar to eutrophication, but with longer lasting or permanent affects as metals are harder to collect and remove than organic biomass from water systems and are in general more toxic to humans and animals at similar concentrations.

1.1.7. *Invasive species*

Another act of unpredicted consequences has been the spread, either intentional or accidental, of foreign organisms to habitats far from their original location. The practice of botanical replanting, as gardens or house-plants were a common occurrence throughout human history, with Roman-culture planting grape varieties wherever they resided (This *et al.*, 2006). While the movement of most plants have not been considered harmful to local ecosystems, some concerns have arisen after two features became evident. First, if the organism is left untended and proliferates, it can significantly impact the ecosystem by consuming most nutrients, or occupying key ecological space, thereby starving out the natural residence and potentially diminishing natives to extinction. Second, if its introduction and spread cannot easily be managed, and removal is difficult, the effect on local ecosystems will be hard to mitigate. A combination of both traits is described as an invasive species, which are not limited to plants but include many other creatures. Unfortunately, freshwater systems have been affected negatively by the addition of various invasive species. Three such examples are the spiny water flea, zebra mussel, and Asian carp (Cooke and Hill, 2010). Both flea and bivalve were introduced to North American lakes and rivers by accidental measure. Likely their adult, egg or larval forms attached to human tools, boats or recreational watercraft, brought them from Europe over the Atlantic, introducing each to new habitats here. Alternatively, Asian carp were purposely introduced into the United States for sport and food fishing. In each case, the organisms flourished once in North American freshwaters, and have been uncontrollably spreading from rivers to lakes. The flea has caused reduction to local fish populations, which were not tolerant to such parasites, while the mussels and carp have been shown to significantly consume nutrients or plankton to the point that their presence can starve out other fish competing for the same food (Karatayev *et al.*, 2015). Asian carp have further been suggested to prey on indigenous fish eggs and larvae, diminishing their numbers drastically (Zhang *et al.*, 2016).

Such negative effects have been concerning when considering that unique species of North American lakes are desirable for a few reasons. As an example, certain fish are popular as human food, and their extinction would be unwanted. Speaking biologically, it is considered an advantage to have wide genetic variability on the planet. If all lakes become monoculture-like under human influence, the lack of diversity may put the remaining species at risk, if a significant disease or environmental change occurred that could eliminate the entire homogenous group. In comparison, diverse capabilities would allow some populations to survive such unforeseen events, preserving life in its entirety.

1.1.8. Anthropogenic environmental change

This brings us to the final concern, one of global magnitude. Modern consensus is that the Earth is warming at rates alarmingly higher than estimated for previous periods in geological records, predicted at 0.15–0.6 °C per decade in the 21st century (Houghton, 2005). Global warming of at least 1.5 °C is suggested by 2100 (Pattyn *et al.*, 2018), quickened by the melting of glacier ice (Wunderling *et al.*, 2020). It can be speculated that our planet is steadily coming out of an iceage, which could be the major cause of global warming trends, but the impacts of human civilization are also of significant levels and may be the strongest influence for drastic changes. While the future can only be predicted based on the information that we currently have, the effect of increasing CO₂ and other greenhouse gas emissions at amounts much higher than in geological records may bear consequences. Regardless of how, the average increase in temperature will likely affect many ecosystem dynamics (Scheffer *et al.*, 2001), with high probability of damaging many biological reservoirs (Albouy *et al.*, 2020). Whether it be the raising of sea level, or the increase of storms and precipitation, the whole world will have to adapt to the changing environment. We will likely witness the disappearance of species that make room for organisms that can thrive in the modified habitat.

However, even with human impact, ecosystem transformation will take time, and requires thorough observation. Declining fish populations (Alfonso *et al.*, 2020) or agricultural crop yield (Zandalinas *et al.*, 2021), have been attributed to aspects of global change and require additional followup, since causation and adaptability remain questions to be answered. The related developments are difficult to evaluate as not every season is the same, where precipitation, average daily temperatures, and other parameters can be drastically different year to year. Some seasons are recognized as severe drought, while others are incredibly wet compared to average. In addition, high-latitude and temperate geographical regions have experienced intermittent freezing temperatures less frequently, with predicted negative consequences (Pi *et al.*, 2021).

These abnormalities are important to distinguish here, as it clearly reveals that many factors need to be accounted for when attempting to understand ecosystem dynamics. Likely, the change of a single attribute can have significant impact on the habitat if provided over a period of time and at a detectable and relevant level, but it requires extended measurement together with many other records to determine its influence.

1.1.9. Lake Winnipeg

The importance of lakes and issues they have faced, lead us to the analysis of a particular aquatic reservoir, Lake Winnipeg (Fig. 1.1A). Located in the same Canadian province as the University of Manitoba, it has many key features that are unique and of great interest. This freshwater lake is the 11th largest in the world with a vast surface area of ~25,000 km², yet is classified as only 25th by volume, rendering the lake considerably shallow when compared to others. North of the 49th parallel and within the cold continental climate zone, it is subjected to drastic temperature changes and a frozen surface for over five months of the year, yet is warm during the summer, reaching 21.5°C (Kottek *et al.*, 2006; Zhao *et al.*, 2021). These combined features result in a lack of chemocline or thermocline due to the well-mixed and shallow nature, dissimilar to most other large lakes, while still having stark seasonal changes. In addition, the lake also has the 3rd largest drainage basin in the world, at nearly 1,000,000 km², spanning from the Rocky Mountains East, encompassing the entire Canadian prairies area, 4 provinces and 4 USA states (Fig. 1.1B). Most is used for agricultural, urban or industrial purposes, and all the associated runoff drains into the lake prior to leaving via the Nelson River, which currently has 5 hydroelectric dams retaining waters for electric energy generation. From both verbal and written sources, this large system has been historically and currently used by Canadian indigenous populations and recent settlements as a socially important waterway, as a drinking source and for its fisheries (Ulrich *et al.*, 2016).

While essential to maintain as a biological resource, growing concerns have been arisen for water quality and habitat sustainability due to the visible changes over the last century (MWSD, 2011). At one point, its vast sandy beaches on the South shore were held in very high regard for tourism, since the oligotrophic lake was clear and healthy. Some living community members still remember safely drinking unprocessed lake water within their lifetime in the vicinity of Gimli in the Sourth Basin, and this possibility was slowly moved North to Gull Harbour, then to Pine Dock, later to Grand Rapids, and now it is unsafe everywhere. Currently, most of the lake is incredibly turbid, ruddy brown with amassed green particulate midsummer.

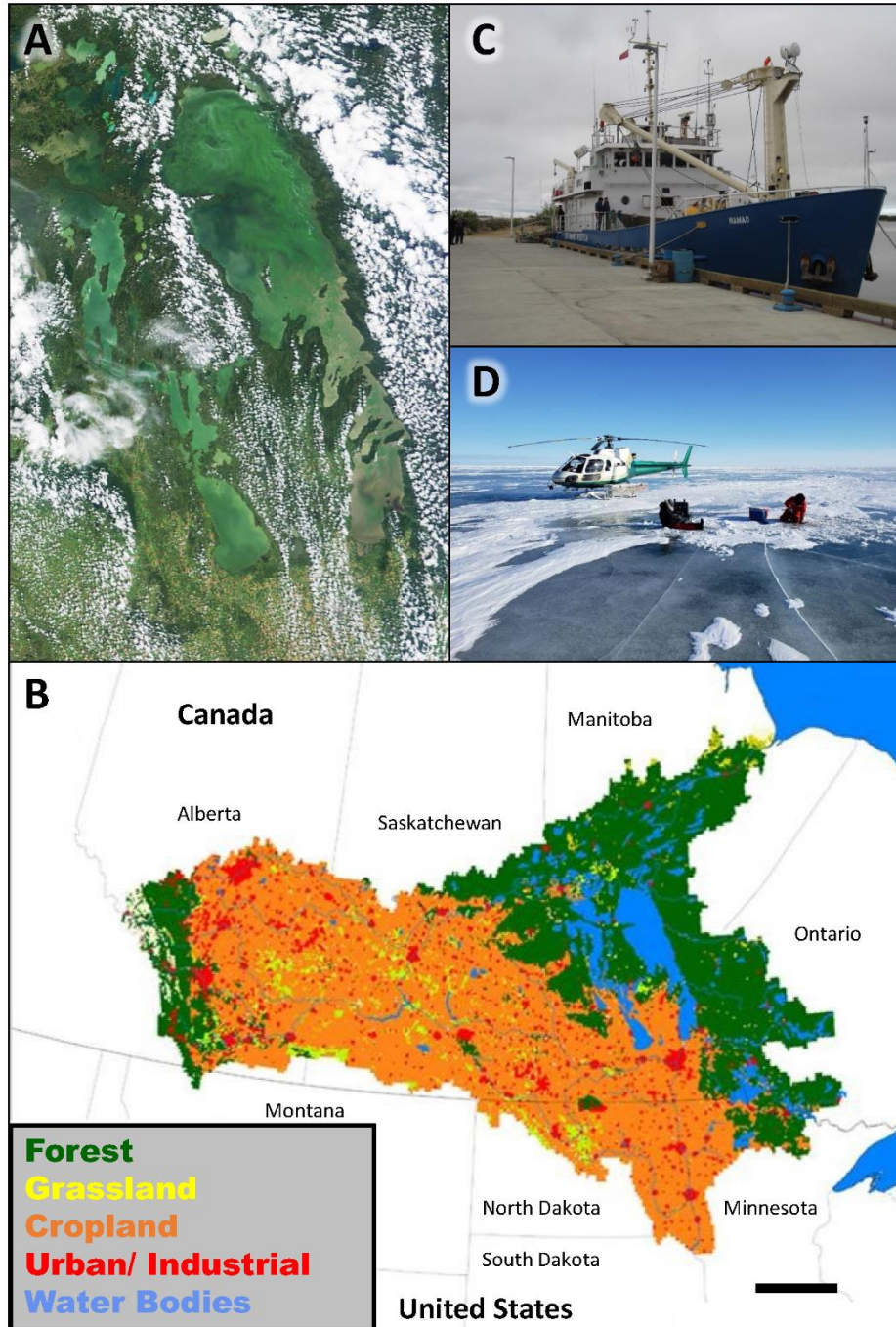


Fig. 1.1. Lake Winnipeg ecosystem and pollution monitoring. (A) August 10th 2015 satellite image showing large emerald algal and cyanobacterial blooms. (B) Drainage basin map separated into land usage zones, predominantly human agricultural and urban areas. Bar, 200 km. (C) M.V. *Namao* operated by Lake Winnipeg Research Consortium. (D) Helicopter access in winter by the Water Science and Watershed Management Branch, Department of Environment, Climate and Parks, Government of Manitoba. Lake Winnipeg Images graciously provided by Dr. K. Scott and the LWRC, in partnership with the Rapid Response Imagery from the Land Atmosphere Near-real time Capability for EOS (LANCE) system operated by the NASA/GSFC/Earth Science Data and Information System (ESDIS).

These changes have been attributed to several influential events. First, the large drainage basin has made the lake susceptible to substantial floods, the most recent significant crisis having occurred in 1950 and 1997. Both massive over-flows rerouted the Red River outlet, the major contributor of nutrients to the lake (Donald *et al.*, 2015), which no longer slowly filters through lowlands and wetlands, but instead now goes directly into the South Basin. It is believed that the lack of residence time in lagoons and marshes has caused much of the clay particulate turbidity, as such sediments are easy to stir up and slow to precipitate.

Second, the agricultural boom across much of the prairies within the drainage basin (Fig. 1.1B), and the modern intense use of fertilizers to keep up with high food demands, are believed to be among the significant sources of runoff causing lake eutrophication (Bunting *et al.*, 2016). Furthermore, most of the largest cities reside on the major rivers that feed the lake, including Winnipeg, which pumps its wastewater effluent directly into the Red River. The antiquated water treatment plants of this city have been linked as a significant contributor of organics and nutrients that flow into Lake Winnipeg (Schindler *et al.*, 2012). Likely a result of the metropolis proximity in addition to its outdated filtration techniques, where the flow and retention time in the Red River does not diminish downstream affects prior to entering the waterbody. In comparison, Edmonton and Calgary may also be producing similar levels of nutrients in their effluent, but their distance from the lake likely mitigates most influence on eutrophication. While both Albertan cities are within the drainage basin at the head of two Saskatchewan rivers, some nutrient effluent is absorbed in the riverbeds prior to flowing into Lake Winnipeg (Sophie *et al.*, 2021).

Regardless of where nutrients are coming from, it is evident that Lake Winnipeg is suffering from eutrophication, and in 2013 was classified as the “Threatened lake of the year” by the Global Natural Fund (Norman *et al.*, 2020). This unfortunate status has not changed since then, as it is still supplied with excess nutrients which are not being filtered out due to the unrestored wetlands and lowlands. Since an increase in organics has been linked to diminished water quality and fish populations, Lake Winnipeg has been monitored more seriously after the great flood of 1997, with the Lake Winnipeg Research Consortium (LWRC) Inc. founded in 1998 to facilitate scientific analysis. Sampling has been conducted across both basins in all primary seasons at limnetic and near-shore zones using research vessels in open-water months of spring, summer and fall (Fig. 1.1C), or bombardier/helicopter in the winter (Fig. 1.1D). Since then, two major scientific special issue publications have focused on Lake Winnipeg (Koehler *et al.*, 2021; Wassenaar and Rao, 2012), culminating in two overarching government-facilitated reviews (MARD, 2022; MWSD, 2011). In brief

summary, fish populations have been monitored predominantly (Enders *et al.*, 2021), in addition to chemical content including dissolved phosphorus and nitrogen as known culprits of nutrient runoff. While a few more recent studies have looked into different zooplankton (Hann and Salki, 2017), or even microplastic contamination (Anderson *et al.*, 2017), the only microbiological works have been either chlorophyll *a* (Chl *a*) measurements to estimate oxygenic phototrophs such as cyanobacteria and algae (Binding *et al.*, 2018), or of coliform counts around recreational sandy beaches for generalized water quality controls. No other bacterial study had yet been done before this PhD program.

1.2. Lake Bacteria and Their Study

It has been well understood that microorganisms inhabit freshwaters since the earliest days of microscopy (Dobell, 1923). Pond samples have been viewed and marveled by their contained complexities of life and morphological diversity, from diatom variants down to the multiplicity of bacterial arrangements (Dobell, 1923; Lane, 2015). Yet, as the science of microbiology advanced, the ecology of habitats has progressed in waves rather than by steady development. Accurate representations of all microorganisms living at specific locations has been difficult to achieve, with many methods having different limitations (Locey and Lennon, 2016), while it has also been hard to fully appreciate the intricacies of interactions and biological processes occurring between these small creatures. Nevertheless, information has been gathered, and a variety of modern techniques, especially when used in combination, have advanced the field of lake microbial ecology (Faust and Raes, 2012). As freshwater bacteria are numerous and varied in many aspects of physiology and phylogeny, independent studies have had to look into three main fields: Diversity, capabilities, and enumerations.

1.2.1. Vast microbial diversity

First by the microscopy of differently shaped single cells and morphotypes (Henrici, 1933), then via the isolation and purification of individual strains by cultivation (Spring *et al.*, 2000), many unique bacteria have been determined in lakes. Such studies were the keystone to understanding each new species and provided possibilities to differentiate and identify bacteria. Later cultivation-independent techniques including fluorescence microscopy (Jürgens *et al.*, 1999), or DNA sequencing (Newton *et al.*, 2011), have shown that many microbes are present in water samples that are yet to be cultivated in a laboratory setting (Yadav *et al.*, 2019).

In recent times, genomic analysis of microbial communities helped to assess a rich volume of information without the need of cultivation, allowing for the accounting of most bacteria present using 16S rRNA gene sequencing. Universal probes and platforms like Illumina MiSeq produce identifiable fragments of many genomes within complex natural samples (Newton *et al.*, 2011; Nevers *et al.*, 2020). Currently, it is estimated that the Earth is home to as many as 1 trillion microbial species (Locey and Lennon, 2016), with far less than <1% of all bacteria isolated and cultivated to date. Nonetheless, average community compositions of lake waters have been tallied (Newton *et al.*, 2011), with members of *Actinobacteria*, *Proteobacteria*, *Bacteroides*, *Cyanobacteria* and *Verrucomicrobia* regularly present in freshwater. However, when these broad phyla are split and individual species considered, most lakes have been shown to contain varied representation. This dissimilarity may be because of differences in sampling methods that include: unaligned factors of location or time, chemical concentrations between lakes, or due to disparity in biological food webs, which can largely influence the microbial structure of each habitat. The lack of bulk environmental data such as water chemistry, physicality, or of other biotic components including bacterial predators, in tandem with microbial diversity analysis, has thus remained as a knowledge gap in the modern study of bacterial ecology in lakes (Nevers *et al.*, 2020), whereas it has provided great benefit to the analysis of smaller habitats such as hot water springs (Power *et al.*, 2018).

1.2.3. Microbial abilities

After their early discovery and confirmation as lifeforms, there has been a great focus to understand what each individual microbe is capable of. Louis Pasteur's initial study of yeasts as the producers of alcohol, and "fowling" bacteria that performed other types of fermentation (Pasteur, 1876), proved to be the first example of specificity in action. Since then, it became clear that different bacteria carry out particular functions.

Within lake habitats, different microbes have also been found to play specific roles (Li *et al.*, 2020). Autotrophs can fix inorganic carbon into organic matter, or make nitrogen, phosphorus and some other elements biologically available. Heterotrophs break down complex organics already available in solution, with some able to digest components that require specific enzymatic mechanisms, such as lignin or cellulose. These organisms are considered consumers and recyclers of organics (Jiao *et al.*, 2010), as they utilize, modify, transfer, and change the existing nutrients. Bacteria occupy a very specific niche in their environment, consuming some molecules, and releasing particular others, while neighboring populations

may then take advantage of the released byproduct for their own processes (Islam *et al.*, 2020). Such consecutive management allows for many defined activities to be the primary biological role of different bacteria, all occupying their own place in the network. While numerous associations are commensal, where organisms metabolically benefit one-another, antagonistic, parasitic, and opportunistic relations also occur as part of the dynamic system (Pradeep Ram *et al.*, 2020). Furthermore, a single bacterium can perform several types of interactions, where it could support a population with the release of one compound, but then prey upon a different neighbor due to the auxotrophic requirement of another auxenic nutrient. This is to say that all microbial relationships are complex, in addition to their associations with multicellular eukaryotes or environmental abiological factors.

Through the study of physiology of purified species, most metabolism, regulation, and responses to external stimulants have been discovered (Overmann *et al.*, 2019). The cultivation of microbes thus provided most of the groundwork essential for our mechanistic understanding of bacterial life, whereas modern applications such as genetics are beginning to contribute new comprehension of organisms unable to grow in laboratory settings. By sequencing partial genomes or MAGs (metagenome assembled genomes), the theoretical abilities of unculturable species have been proposed based on their homologous fragments to known enzyme genes (Frioux *et al.*, 2020). Microbes that have been elusive thus-far in culture can then hopefully be purified in the future with this additional understanding. If successful, we may eventually glean even more metabolic information once the remaining 99% of environmental bacteria become culturable (Cross *et al.*, 2019). Compiling all related discoveries, it is obvious that various microorganisms are involved in different biogeochemical cycles by being the predominant forces responsible for specific transformations.

1.2.3. Cellular enumeration

Knowing the number of bacteria and other microbes in an area has always been a critical part of understanding their related importance. This is of great interest once the abilities of a particular species have been sorted out, and there is a desire to know its impact to the ecosystem. In general, microorganisms make up a significant biomass in lakes based on counting all cells with DAPI-staining microscopy, culture-based enumerations (Bussmann *et al.*, 2001), or via modern quantifications based on qPCR (Jiang *et al.*, 2009) and flow cytometry (Brown *et al.*, 2019). Even though microscopy has remained useful since the early days, it is still difficult to exercise reliably due to environmental samples having larger particles, debris, and heterogeneous elements that interfere with computer imaging (Koblížek, 2015). Cellular counts received

from cultivation have been useful as they allow for the enumeration of organisms with particular growth requirements. This can be advantageous when identifying the number of microbes that make up a whole group with broad metabolic capabilities (such as all heterotrophs or anaerobes). This method is also useful to count how many microbes have a specific tolerance to a pollutant (a metal or biological toxin for example). Other techniques to identify the presence of a distinct group include qPCR which can be quite useful to determine how many copies of a specific gene are within a defined volume of sample. While this does not require cultivation, it is unfortunately limited to quantification only, where the purification of isolates can provide additional opportunities to check various growth capabilities.

Regardless, lake bacteria have been frequently counted, with microscopic values of totaling around 10^5 - 10^6 cells/mL (Fauteux *et al.*, 2015; Hobbie *et al.*, 1977), while cultivated heterotrophs have averaged 10^4 - 10^5 CFU/mL in water (Frischer *et al.*, 2000). Both values were highly dependent on the environment, where seasonal progression and the changing of temperature, or other parameters, significantly effected bacterial presence. When separated into particular functional groups or species, individual bacteria have been recorded with independent ebb and flows (Pernthaler *et al.*, 1998). As this is the case, much information is still needed to understand how certain groups change in numbers, if it is based on spatial or temporal patterns, and why they fluctuate: Is it due to chemical, physical or biological reasons?

1.2.4. Combined analytical approach

While the three components: Bacterial diversity, capability, and numbers are individually important, few microbiological studies have taken into account all of them simultaneously as a tremendous amount of work is required to factor in several features. Yet, this has been precisely necessary to understand how microbes fit into the ecosystem (Overmann *et al.*, 2019). While several current studies have compared the microbial community diversity to few environmental parameters, with once example observing 60 lakes from a single timepoint (Sadeghi *et al.*, 2021), even such impressively comprehensive analysis lack the revelation of bacterial activity in each location. Such an over-encumbrance is likely why microbial ecology is a relatively difficult and highly nuanced science. In general, many current environmental questions require “big-data” science, or the simultaneous analysis of many factors, such as following an allinclusive group of species rather than only the one of interest, in addition to detailed water chemistry and temporal sampling, to obtain clarity on how your research curiosity fits into the big picture. Therefore, while it is known that certain species fix nitrogen or carbon, and others are acetogens, methanogens, heterotrophs or autotrophs, and an individual

functional group can be tracked (Yang *et al.*, 2020), the study of one groups' niche in waterbodies is ideally observed in addition to other related populations.

1.2.5. Bacteria within the lake foodweb

Considering the high numbers of bacteria in freshwater, and their ability to produce and transform organics, it is clear that they play a significant role in nutrient cycles. Acting at the base of the foodweb, their incorporation of simple chemicals or degradation and conversion of organics from deceased cellular material, has made them either good candidates for predation, or reasonable partners for symbiosis in nature. Despite many zooplankton and filter feeders historically presumed to digest dissolved “nutrients”, recent studies are beginning to look into consumption of bacteria (Frischer *et al.*, 2000; Garcia-Chaves *et al.*, 2015). Some works even found selective grazing on certain prokaryotic species, and preference against toxin producers. While older documents have suggested dead cells and released compounds were the organic preference (Horgan and Mills, 1997), some modern work has reported bacterivores to prey upon live microbes (Mezzanotte *et al.*, 2016). Although either may be true, the lack of live/ dead ratios in most analysis leave such confirmations to be still drawn. In any case, associations can also include commensal cooperation of bacteria with higher forms of eukaryotes. A few aquatic examples include symbionts of amoeba (Dirren and Posch, 2016), zooplankton (Chernitsyna *et al.*, 2021), or Porifera (Latyshev *et al.*, 1992).

Nevertheless, the biogeochemical impact of microbial communities is still ongoing research, as the interactions of bacteria with other representatives in aquatic environments is the least studied aspect of lake bacterial ecology. Since Lake Winnipeg has several filter feeding mollusks, zooplankton, and fish populations (MWSD, 2011), and these individual populations may have key associations with or dependence on bacteria, they clearly warrant further investigation.

As there is still much to be gleaned on lake microbial compositions, it is thus important to focus on a few particular groups and identify their contributions and influences within the environment. Considering some of the most prevalent and functionally important bacteria (Newton *et al.*, 2011), focus can be drawn on those that perform photosynthesis.

1.3. Microbial Photosynthesis

The study of photosynthesis began as a subject of botany, with later consideration of microscopic unicellular algae and cyanobacteria that were historically considered as simple plants, with the later

originally described as blue-green algae (Hohmann-Marriott and Blankenship, 2011). From the very beginning, it was clear that each of these organisms were unified by their use of sunlight energy for growth, and without illumination they would perish.

1.3.1. Light mediated biological energy production

There are several logically supported theories that light-mediated biological energy production has been a fundamental part of life's evolution on the planet Earth, as it is a more efficient method of synthesizing adenosine triphosphate (ATP) when compared to anaerobic fermentation or lithoautotrophic means, in order to create organic materials from inorganics (Hohmann-Marriott and Blankenship, 2011). The main type of light-to-chemical-energy conversion involves chlorophyll (Chl) or bacteriochlorophyll (BChl) based photophosphorylation, called photosynthesis (Hohmann-Marriott and Blankenship, 2011). Rhodopsin dependent production is another strategy to absorb light first found in marine archaea (Stoeckenius *et al.*, 1979), and more recently in various aquatic bacteria (Rozenberg *et al.*, 2021), but is significantly less energetically efficient, producing less ATP per lumen of light.

1.3.2. Chlorophyll and bacteriochlorophyll based photosynthesis

Focusing on photosynthesis, it is a pathway that involves the conversion of light into biological energy, ATP, which is used as a universal biochemical compound that powers all metabolic reactions (Nirody *et al.*, 2020). The process begins when photons excite a chromophore, a small molecule with numerous conjugated double bonds that confer the ability to absorb high energy such as light. In this case, the chromophore is either Chl or BChl, both of which consist of ~900 Da structure that includes a core porphyrin ring typically with an imbedded Mg cation, and various side chains that differentiate pigment types (Chl *a-d* and *f*, or BChl *a-g*). Chlorophylls are bound to a protein reaction center (RC) that contains a special pair of Chl or BChl in addition to a few of their precursors pheophytin (Pheo)/ bacteriopheophytin (BPheo). Most RC are surrounded by light harvesting (LH) subunits, which also have Chl or BChl and small proteins. The entire complex acts as an antenna to deenergize photons, funneling electrons (e⁻) to membrane-bound quinones (Q), and into the e⁻ transport chain. This can then power a cytochrome bc₁ complex, or similar system that generates a proton gradient across the membrane, that activates ATPase. These e⁻ can continue cycling to cytochromes, which in some species can also be bound to the RC, returning e⁻ back to the system, creating a cycle of e⁻ flow. Furthermore, many organisms capable of photosynthesis contain auxiliary pigments, called carotenoids bound into their LH and RC that may additionally absorb different wavelengths of light and help

transfer extra energy to the RC for increased ATP production efficiency (Hohmann-Marriott and Blankenship, 2011).

This generalized system, as presented by mentioning either Chl and BChl, has been naturally evolved into two fundamental strategies, that of oxygenic or anoxygenic photosynthesis.

1.3.3. *Aerobic oxygenic phototrophs (AOP)*

Organisms that perform photosynthesis with Chl bound into their RC perform both cyclic and non-cyclic e- transport by two different photosystems (PS), PSI and PSII, respectively (Fig. 1.2B) (Lea-Smith *et al.*, 2016). By containing Chl *a* or *b* most commonly, their RC absorb light near 660-700 nm (Fig. 1.2A), contributing to a greenish appearance. The redox poise of their RC components require H₂O as an external e- donor, which is photolysed by PSII and generates e- for PSI, while also creating oxygen as a byproduct, hence their name “oxygenic”. All single-cellular AOP fit into one of two categories: Prokaryotic cyanobacteria or eukaryotic algae (Hohmann-Marriott and Blankenship, 2011).

While most algae contain primary and secondary pigments that maintain their green appearance, cyanobacteria often have phycobili proteins as auxiliary light-harvesting components that attribute a bluish hue, awarding their “blue-green” root in nomenclature, hence “cyano-bacteria”. Cyanobacteria are monophyletic within the Bacteria domain, whereas algae are in general clustered in one branch of the Eukaryota domain on the tree of life, but are defined in several groups under botanical taxonomic codes (Wehr *et al.*, 2015). Recent works have attempted to reorganize cyanobacteria within bacteriological systematics, which rely on the polyphasic approach using chemotaxonomy and phylogeny (Oren *et al.*, 2021), but this phylum still requires additional reclassification using phylogenetic and chemotaxonomic approaches to be inline with other bacterial clades. Independently, algae adhere to botanical codes and roots, with some modern partial rearrangements that have relied on information received from molecular genetic studies (Bhattacharya and Price, 2020). Even though improvements have been achieved to classify these microbial eukaryotes, their genome polyploid nature has slowed the progress of algal phylogenetic advances when compared to prokaryotic cyanobacteria (Bhattacharya and Price, 2020).

Regardless of taxonomic restructuring, most AOP share many common traits, which include their oxygenic photosynthesis linked to autotrophy via the fixation of carbon dioxide. This type of photoautotrophy has been responsible for the majority of organic nutrient production from inorganics on the planet, and its

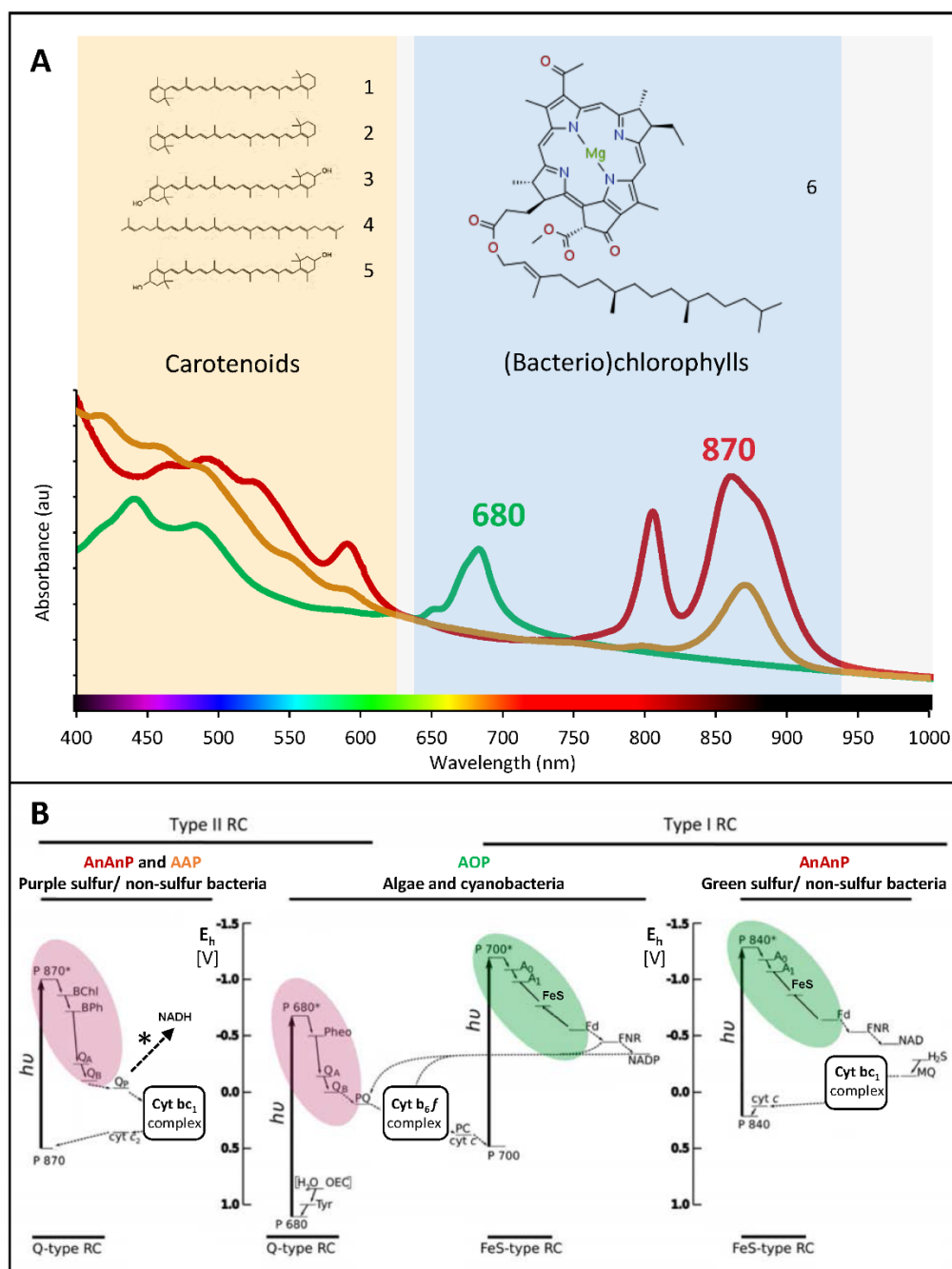


Fig. 1.2. Absorbance spectra and photosynthetic e- transport. (A) Whole cell spectra of AOP (green), AnAnP (red), and AAP (orange). Maxima from carotenoids (yellow) and bacteriochlorophyll (blue) with example pigment structures. (1) α -carotene (2) β -carotene (3) Lutein (4) Lycopene (5) Zeaxanthin (6) BChl a. **(B)** Generalized e- transport cycles of reaction centers (RC) and the related components of each photosynthetic group. Modified from (Hohmann-Marriott and Blankenship, 2011). Type II RC: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Pheo, pheophytin; QA , primary electron accepting quinone; QB , secondary e- accepting quinone; Cyt, cytochrome; *, reverse e- flow. Type I RC: A_0 , primary e- acceptor; A_1 , secondary e- acceptor; FeS, RC-associated iron sulfur clusters; Fd, ferredoxin; FNR, ferredoxin-NAD(P) oxidoreductase.

O₂ byproduct has been linked to the oxygenation of the Earth's atmosphere (Hohmann-Marriott and Blankenship, 2011). While algae also produce RuBisCo enzyme and can fix CO₂, nitrogen fixation is a capability of only prokaryotic cyanobacteria. Furthermore, both the cyanobacteria and algae typically solely rely on their photosynthesis for energy, with few capable of growth in the dark when supplied with simple sugars for glycolysis (Mannan and Pakrasi, 1993).

The self-sustainability and lack of dependency on other organisms for nutrients has rendered AOP a capability to grow all over the planet, wherever light and water are available (Gaysina *et al.*, 2019). Vast numbers exist in the open oceans, established as the major producers of O₂ rather than land plants (Andreeva *et al.*, 2020). They also inhabit terrestrial sediments and freshwaters, but the various ecosystems in these terrains make enumeration and influence harder to quantify, and still requiring analysis. Rate-limiting factors to AOP growth and survival include the available metal concentrations, such as Fe and Zn, which are biologically important for enzymatic processes, e- transfer during respiration and photosynthesis, and nucleic acid or chlorophyll synthesis (Marsh *et al.*, 1963). However, most metal cations are sparse in nature, particularly in aquatic habitats as they are inherently insoluble. As these organisms also rely entirely on photonic and not chemosynthetic energy, their growth is also contingent on overall available heat, where cold waters will slow biological processes (Robarts and Zohary, 1987), and too much warmth can disrupt and damage activities, having a tolerance range between 1-74 or 1-57°C for prokaryotic and eukaryotic AOP, respectively, with all preferring 15-35°C (Seckbach and Oren, 2007).

While taxonomic classifications require more work, there is much still to learn regarding their physiology. In Lake Winnipeg, the most information about any microbial community member has been for AOP via the determination of Chl a levels, typically collected from spectroscopic means of quantification. Their enriched presence, and proliferation has been directly linked to eutrophication, particularly from fertilizers such as nitrogen and phosphorus loading as these two elements are typically the natural limiting factors for the growth of AOP (Qin *et al.*, 2013). Detecting emerald green turbidity of waters is a direct and easily quantifiable method to examine how nutrient loading is affecting a water system, hence the successful long-term use for AOP analysis within Lake Winnipeg. So far, Chl a has been recorded over the past few decades from 4.1 to 35.0 µg/L, averaging 12.2 µg/L mid-basin (MARD, 2022). Seasonal measurements have revealed mid-to-late summer peaks, where concentrated biomass were sporadically observed in some years. Since cyanobacterial blooms have a tendency to aggregate and do not evenly disperse into the water column,

the inconsistent sighting or sampling of these masses in Lake Winnipeg suggests that even with its well-mixed nature, some microorganisms may only be accurately estimated after numerous thorough surveys.

In addition to Chl measurements, one researcher, Dr. Hedy J. Kling has been monitoring cyanobacterial and algal species in Lake Winnipeg via classical microscopic morphological identifications over the past 20 years, while also analyzing the microfossil records. Her findings have suggested that *Stephanodiscus* and *Aulacoseira* are the two major pelagic diatom genera present in the lakes history (Kling, 1998). Furthermore, approximate quantification has shown that *Aphanizomenon* and *Dolichospermum* are the main contributors to blooms (McKindles *et al.*, 2019). These initial findings deserve additional follow-up study using modern techniques to confirm the species composition, enumerate numbers, and see how they fluctuate in the ecosystem.

1.3.4. Anaerobic anoxygenic phototrophs (AnAnP)

In comparison to AOP investigated as early as the 1600's by Antonie van Leeuwenhoek (Dobell, 1923) or Carolus Linnaeus in the 1700's (Jarvis, 2016), anoxygenic phototrophs were described more recently, yet still in the distant past, likely first by Sergei Winogradski in the late 1890's (Dworkin and Gutnick, 2012). Since then, this group has been explored quite vigorously, particularly in the early 20th century as model organisms of prokaryotic physiology. First split into two major groups, purple and green bacteria based on most typical colourations (due to both BChl and carotenoid content), they were later shown to have several coordinated traits. This included particular RC, the incorporation of specific BChl molecules, and e⁻ carriers matching required redox potentials (Fig. 1.2B). A biological energy production system only found in bacteria, these organisms strictly perform photosynthesis anaerobically, and do not produce oxygen as a byproduct, hence anoxygenic. Primary external e⁻ donors that feed into their systems are usually sulfide or thiosulfate, rewarding each group with a name, as in "purple sulfur" or "green sulfur" bacteria. Some species of each group, but predominantly of purple bacteria, cannot tolerate sulfide at high concentrations and instead use different e⁻ donors such as partially reduced acetate, malate, or other non-sulfur components, extending the name combination to purple nonsulfur bacteria (PNSB).

While these organisms are linked by their requirement for anaerobiosis to photosynthesize, the anoxygenic phototrophic bacteria as a whole have a diverse arrangement of metabolic pathways, exemplified by *Rhodospseudomonas palustris* and its large variety of capabilities (Larimer *et al.*, 2004). To highlight their activities (George *et al.*, 2020), several AnAnP can grow photoautotrophically, obtaining energy from light

and creating organic components by fixing inorganic carbon and nitrogen. In addition, a large proportion can also grow photoheterotrophically, still obtaining energy from light, but without expending energy to synthesize organics, and instead perform anabolism by acquiring compounds from the surroundings. Moreover, some examples among the purple non-sulfur bacteria can even grow entirely heterotrophically aerobically, foregoing photosynthesis for chemosynthesis in the presence of oxygen, or anaerobically by fermentation, and respiring on nitrates.

Such metabolic versatility has revealed AnAnP to be found in many ecosystems (Kushkevych *et al.*, 2021), but they are typically predominant members when oxygen is absent. Since the Earth's atmosphere is oxic at 21% O₂, AnAnP reside most in sediments or deep unmixing waters that have formed chemoclines with oxygen depletion zones, but maintain light penetration.

After initial descriptions from microscopy, physiology and chemotaxonomy, the recent use of phylogeny has supported much of the metabolic coherency found among AnAnP, suggesting anoxygenic photosynthesis to be a long vertically descended trait (Imhoff *et al.*, 2019). Likely originating 3.5 billion years ago (Hohmann-Marriott and Blankenship, 2011), AnAnP now differentiate into key clades of Prokaryotes. A current list of recognized type species and their respective hierarchical positions (Table 1.1) reveal green sulfur and non-sulfur bacteria to be among *Chlorobiota* and *Chloroflexi*, respectively, whereas all purple sulfur and non-sulfur species are among specific Proteobacteria clades, and the exceptional gram-positive *Heliobacteria* that contain BChl *g* are uniquely placed among the *Firmicutes* phylum.

Although AnAnP received a great deal of attention in the early and mid-20th century, their investigation has been lagging behind in the 21st (Kushkevych *et al.*, 2021). Likely due to the shift of focus in microbiology towards antibiotics and infection, they have not been the focus of multi-omics approaches as much as medical pathogens. Hence, a lot of what is known about transcription, translation, and regulation of cellular pathways has come from the now well studied heterotrophic *E. coli* and *P. aeruginosa*. Internal mechanisms are speculated to occur similarly in different prokaryotic phototrophs, but many genera still require experimental confirmation. *R. palustris* is one example of a AnAnP that has been well documented for its metabolism (Pechter *et al.*, 2015; Cogan, *et al.*, 2018; Fixen *et al.*, 2019), and is thus the exemplary PNSB. However, other forms of AnAnP including dedicated anaerobic purple and green sulfur bacteria do not have thoroughly described model organisms. Thus, there remains a great deal still to be gleaned when studying

these exciting organisms in physiology both from the point of a modern omics approach and via ecological surveys using current technology.

Bacterial populations of Lake Winnipeg have not been thoroughly analyzed, but its physical chemistry can offer some hints towards what organisms could be present. The relative shallowness that prevents chemoclines is unique among all great lakes (Brunskill *et al.*, 1980; Tellier *et al.*, 2022). Permanent oxygen penetration to the benthic zone would likely promote aerobic microorganisms. Therefore, while most strict anaerobic phototrophs would be theoretically inhibited, the assimilatory and dissimilatory multifunctionality of some AnAnP described above, could mean they are present in the lake. These bacteria are infrequently enumerated in euphotic zones of freshwater habitats, being detected predominantly at chemoclines only (Imhoff, 2017), as they are presumed absent in the oxic locations. However, such confirmations would be necessary when comparing each population. Sampling littoral sediments that are within range to be illuminated could yield AnAnP, as the oxygen cannot penetrate deeply into the solid bottom (Imhoff, 2017), and thus anaerobes can be present. Indeed, sandy shoreline sediments are one of the few areas in Lake Winnipeg tested for the presence of bacterial coliforms (MARD, 2022). As facultative heterotrophic anaerobes, they indicate fecal contamination and unhealthy water quality for human recreation or consumption. During a few instances, coliform counts have suggested unsafe levels in sandy shores, but these results never persisted. Further, the sporadic detection might be due to aquatic avian species rather than human waste (Whitman *et al.*, 2014). Since common methods of counting do not apply modern microbial identification techniques, the actual risk of pathogens is debatable. What is not questionable however, is the discovery of facultative anaerobes in Lake Winnipeg shoreline sediments, suggesting that bacterial phototrophic populations may exist, thus warranting further investigation.

1.3.5. Aerobic anoxygenic phototrophs (AAP)

So why search for anoxygenic phototrophs in euphotic oxic waters, if AnAnP perform this biological-energy synthesis only as an anaerobic process? In contrast to AnAnP, strict AAP have been discovered the most recently of all phototrophs during the late 1970's first with a marine species *Erythrobacter longus* producing BChl *a* aerobically (Harashima *et al.*, 1978; Shiba and Simidu, 1982), and then *Sandaracinobacter sibiricus* from freshwaters (Yurkov *et al.*, 1997; Yurkov and Gorlenko, 1990), confirming their global presence. These strains were shown to have functional RC and LH containing BChl *a* similar to that of PNSB

Table 1.1. Current list of AnAnP type species.

Phylum	Class	Order	Family	Genus	Species	Reference
Chloroflexi	Chloroflexia	Chloroflexales	Chloroflexiaceae	Chloroflexus	aurantiacus	(1)
Chloroflexi	Chloroflexia	Chloroflexales	Chloroflexiaceae	Oscillochloris	trichoides	(1)
Chloroflexi	Chloroflexia	Chloroflexales	Chloroflexiaceae	Chloroploca	asiatica	(1)
Chloroflexi	Chloroflexia	Chloroflexales	Roseiflexiaceae	Roseiflexus	castenholzii	(1)
Chloroflexi	Chloroflexia	Chloroflexales	Kouleoethrichinae	Kouleoethrix	aurantiaca	(1)
Firmicutes	Clostridia	Eubacteriales	Heliobacteriaceae	Heliobacterium	chlorum	(3)
Firmicutes	Clostridia	Eubacteriales	Heliobacteriaceae	Heliomicrobium	gestii	(3)
Firmicutes	Clostridia	Eubacteriales	Heliobacteriaceae	Heliophilum	fasciatum	(3)
Firmicutes	Clostridia	Eubacteriales	Heliobacteriaceae	Helioestis	daurensis	(3)
Chlorobiota	Chlorobia	Chlorobiales	Chlorobiaceae	Ancalochloris	perfilievii	(4)
Chlorobiota	Chlorobia	Chlorobiales	Chlorobiaceae	Chlorobaculum	tepidum	(4)
Chlorobiota	Chlorobia	Chlorobiales	Chlorobiaceae	Chlorobium	limicola	(4)
Chlorobiota	Chlorobia	Chlorobiales	Chlorobiaceae	Chloroherpeton	thalassium	(4)
Chlorobiota	Chlorobia	Chlorobiales	Chlorobiaceae	Prosthecochloris	aestuarii	(4)
Proteobacteria	α -Proteobacteria	Hyphomicrobiales	Blastochloridaceae	Blastochloris	viridis	(1)
Proteobacteria	α -Proteobacteria	Hyphomicrobiales	Hyphomicrobiaceae	Rhodomicrobium	vannielii	(1)
Proteobacteria	α -Proteobacteria	Hyphomicrobiales	Nitrobacteraceae	Rhodoplanes	roseus	(1)
Proteobacteria	Alphaproteobacteria	Hyphomicrobiales	Nitrobacteraceae	Rhodopseudomonas	palustris	(18)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Rhodospirillaceae	Phaeospirillum	fulvum	(1)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Rhodospirillaceae	Pararhodospirillum	photometricum	(1)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Rhodospirillaceae	Rhodospirillum	rubrum	(1)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Rhodospirillaceae	Roseospira	mediosalina	(1)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Planktomarina	temperata	(1)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter	capsulatus	(1)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Halovulum	dunhuangense	(1)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Cereibacter	changelensis	(1)
Proteobacteria	β -Proteobacteria	Rhodocyclales	Rhodocyclaceae	Rhodocyclus	purpureus	(1)
Proteobacteria	β -Proteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax	gelatinosus	(1)
Proteobacteria	β -Proteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga	flava	(1)
Proteobacteria	β -Proteobacteria	Burkholderiales	Comamonadaceae	Rhodoferrax	fermentans	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Ectothiorhodospiraceae	Ectothiorhodosinus	mongolicus	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Ectothiorhodospiraceae	Ectothiorhodospira	mobilis	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Allochrochromatium	vinosum	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Caldichromatium	japonicum	(5)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Chromatium	okenii	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Halochromatium	salexigens	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Imhoffiella	purpurea	(6)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Isochromatium	buderi	(7)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Lamprobacter	modestohalophilus	(8)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Lamprocystis	roseopersicina	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Marichromatium	gracile	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Nitrosococcus	nitrosus	(9)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Phaeobacterium	nitratireducens	(10)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Phaeochromatium	fluminis	(11)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Rhabdochromatium	marinum	(12)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thermochromatium	tepidum	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thioalkalicoccus	limnaeus	(13)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiobaca	trueperi	(14)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiocapsa	roseopersicina	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiococcus	pfennigii	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiocystis	violacea	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiodictyon	elegans	(15)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiohalocapsa	halophila	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiolamprovum	pedioforme	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiopedia	rosea	(16)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiophaeococcus	mangrovi	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiorhodococcus	minor	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiorhodovibrio	winogradskyi	(1)
Proteobacteria	γ -Proteobacteria	Chromatiales	Chromatiaceae	Thiospirillum	jenense	(17)
Proteobacteria	γ -Proteobacteria	Chromatiales	Halorhodospiraceae	Halochlorospira	halochloris	(2)
Proteobacteria	γ -Proteobacteria	Chromatiales	Halorhodospiraceae	Halorhodospira	halophila	(2)

(1) (Mehrshad et al., 2018); (2) (Imhoff et al., 2022); (3) (Kyndt et al., 2021); (4) (Imhoff, 2003); (5) (Saini et al., 2020); (6) (Nupur et al., 2017); (7) (Imhoff et al., 1998); (8) (Gorlenko et al., 1979); (9) (Watson, 1965); (10) (Nupur et al., 2015); (11) (Shivali et al., 2012); (12) (Dilling et al., 1995); (13) (Bryantseva et al., 2000); (14) (Rees et al., 2002); (15) (Luedin et al., 2018); (16) (Eichler and Pfennig, 1991); (17) (Imhoff et al., 2021); (18) (van Niel, 1944).

(Fig. 1.2), but surprisingly only produced by aerobic cultures in the dark. Growing AAP at simulated day/night cycles revealed them to assemble BChl into RC and LH at night, which is used for energy production from light the following day (Yurkov and van Gemerden, 1993).

Many AAP have been isolated with striking colony pigmentation, coloured pink, red, orange, yellow, or brown (Fig. 1.3A), due to their predominance of carotenoids that dwarf the amount of RC LH complex's produced (Yurkov *et al.*, 1993). Thus far all have been shown to contain BChl a as their primary pigment for photosynthesis, providing peaks of 750, 800, and ~855 nm from RC, and typically 800-869 nm of LHI (Hughes *et al.*, 2020; Rathgeber *et al.*, 2012; Yurkov *et al.*, 1998). While typical PNSB LHII of 800-850 nm have not yet been discovered in AAP, unusual 800-835 nm LHII was reported for some freshwater species (Yurkov and Beatty, 1998). The redox poise of their RC primary e⁻ acceptor Q_A do not allow anaerobic growth or the use of sulfide as an e⁻ donor, instead preferring acetate similar to some PNSB. Interestingly many morphological varieties of AAP have been found, in addition to red or blue shifted major LHI peaks from 875 down to 855 nm, respectively. Furthermore, some have LHII and other typically bound components (Fig. 1.3B), while others seemingly lack associated LHII, cytochromes, and H protein (Fig. 1.3C), representing a large diversity of PS composition.

Of note, AAP have been discovered entirely as photoheterotrophic organisms, incapable of CO₂ fixation, and all lacking RuBisCo enzyme (Yurkov and Hughes, 2017). Moreover, their photosynthesis contributes up to 30% of their biological energy production in laboratory settings, relying on chemosynthesis for the remainder via respiration (Hauruseu and Koblížek, 2012; Yurkov and van Gemerden, 1993). The lack of autotrophy in AAP has suggested a niche to out compete strict heterotrophs in eutrophic environments while producing ATP by two dissimilatory pathways rather than using only one, or in oligotrophic environments by reserving all organics as building blocks for biosynthesis and anabolism, without using any for chemical energy production, which is instead procured from sunlight.

Over the past 4 decades, AAP diversity and ecology have been investigated, with several important reviews published (Koblížek, 2015; Rathgeber *et al.*, 2004; Yurkov and Beatty, 1998; Yurkov and Csotonyi, 2009; Yurkov and Hughes, 2017). As the last overview was released in 2017, an updated list of all AAP species is shown in Table 1.2. Many groups have been found among Proteobacteria, with new isolates related to *Gemmatimonadetes*. While horizontal gene transfer (HGT) has been proposed as a way of photosynthesis lateral movement between bacteria, particularly among *Rhodobacteraceae* members (Brinkmann *et al.*,

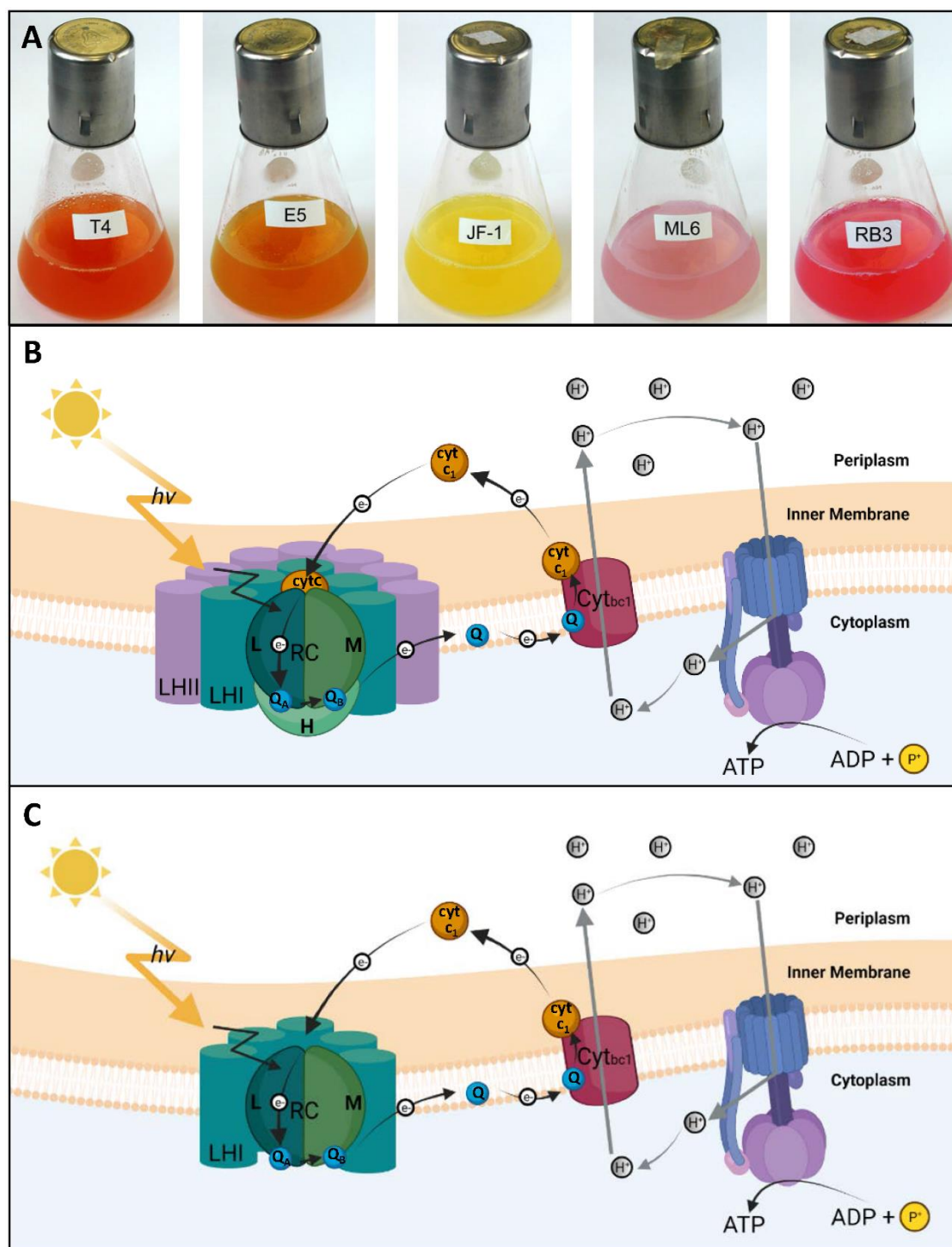


Fig. 1.3. AAP pigmentation and photosynthetic RC composition. (A) Various carotenoid content of AAP strains. *Erythrobacter litoralis*, T4; *Erythromicrobium ramosum*, E5; *Citromicrobium bathyomarinum*, JF1; *Roseicyclus mahoneyensis*, ML6; *Roseococcus thiosulfatophilus*, RB3. (B) Photosynthetic membrane cross-section of LH-RC complexes with bound LHII and cytochromes, or (C) RC lacking auxiliary components for light absorption. RC, L, H, and M, Reaction center subunits; hv, photonic energy; cyt, cytochrome; Q, quinone. Diagrams made in Biorender.

2018), or from *Proteobacteria* to *Gemmatimonadetes*, the recent structural characterization of *Gemmatimonadetes* PS-LHI complex found it to be entirely unknown and unique (Qian *et al.*, 2022), suggesting its own long ancestral lineage. Also, the genetic study for all anoxygenic phototrophs including AnAnP also concluded that AAP photosynthesis genes were equally as delineating as other conserved genes of 16S rRNA (Imhoff *et al.*, 2019). Therefore, current speculation proposes that photosynthesis is predominantly a vertically transferred trait, passed down by decent with some modifications during evolution.

In addition to very wide diversity of species, AAP have been found practically anywhere sampled for, from extreme environments such as hydrothermal vent plums, mine tailings, salt springs, meromictic lakes, biological soil crusts, to mundane environments covering oceans, rivers, and lakes (Koblížek, 2015; Vladimir Yurkov and Beatty, 1998; Yurkov and Hughes, 2017). The harsh environments had received quite a bit of attention in early experiments as being the most unusual habitats, whereas oceans as the largest ecosystem on the planet, were also later studied in detail. Freshwater habitats have only been more recently investigated, and in the past decade have had a few research groups looking into various enumerations in small ponds and a few lakes (Fauteux *et al.*, 2015; Man *et al.*, 2008). The active study of AAP has been due to their massive presence in some ecosystems, where they have made up to 37% of microbial communities, often peaking at 15% among marine or 20% freshwater bacteria (Koblížek, 2015). Culture-based counts have been as high as 3.39×10^7 CFU/mL in extreme environments (Csotonyi *et al.*, 2008), and culture-independent autofluorescent microscopy have suggested numbers up to 5.49×10^5 cells/mL in some lakes (Fauteux *et al.*, 2015). Both the large range in diversity and significant biomass in many ecosystems has strongly supported that AAP have an important role in their habitat, but fine details remain unclear.

To decipher the impact of AAP on biogeochemical cycling, many studies have considered their physiological abilities, with great focus on carbon cycling. The lack of RuBisCo in all known and tested isolates clearly indicated that no significant CO₂ fixation can occur, with only minuscule amounts possible through typical anaplerotic pathways (Tang *et al.*, 2009). Thus, AAP predominantly act in the carbon cycle as aerobes that respire and release CO₂ during oxidative phosphorylation (Piwosz *et al.*, 2022), and use organics as building blocks for their anabolism. While this would appear no different than the effect of strict heterotrophs in the same environment, the additional ATP produced from light energy in photosynthesis helps them to metabolize quicker and more efficiency (Kirchman and Hanson, 2013).

Table 1.2. Updated list of AAP type species.

Phylum	Class	Order	Family	Genus	Species	Reference
Proteobacteria	α -Proteobacteria	Sphingomonadales	Erythronadaceae	Erythrobacter	litoralis	(1)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Erythronadaceae	Erythromicrobium	ramosum	(1)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Erythronadaceae	Porphyrobacter	sanguineus	(1)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Sphingomonadaceae	Blastomonas	ursincola	(1)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	kaistensis	(3)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Sphingomonadaceae	Citromicrobium	bathymarinum	(2)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Sphingomonadaceae	Citrimicrobium	luteum	(6)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Sphingosinellaceae	Sandaracinobacter	sibiricus	(1)
Proteobacteria	α -Proteobacteria	Sphingomonadales	Sphingosinellaceae	Sandarakinorhabdus	limnophila	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseobacter	denitrificans	(1)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseovarius	tolerans	(2)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseibium	denhamense	(2)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Rubrimonas	cliftonensis	(2)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseivivax	halodurans	(2)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Sulfitobacter	guttiformis	(2)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseinatronobacter	thiooxidans	(2)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseicyclus	mahoneyensis	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Thalassobacter	stenotrophicus	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Dinoroseobacter	shibae	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseibacterium	elongatum	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseisalinus	antarcticus	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Labrenzia	alexandrii	(3)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseibaca	ekhonensis	(4)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseiciterium	antarcticum	(4)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Charonimicrobium	ambiphotophilum	(4)
Proteobacteria	α -Proteobacteria	Rhodobacterales	Rhodobacteraceae	Roseibacula	alcaliphilum	(6)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Roseococcus	thiosulfatophilus	(1)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Acidiphilium	rubrum	(1)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Acidishaera	rubrifaciens	(2)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Rubritepida	flocculans	(2)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Craurococcus	roseus	(2)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Paracraurococcus	ruber	(2)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Geminicoccus	roseus	(3)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Humitalea	rosea	(6)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Acetobacteraceae	Roseomonas	aestuarii	(4)
Proteobacteria	α -Proteobacteria	Caulobacterales	Maricaulaceae	Alkalicaulis	satelles	(11)
Proteobacteria	α -Proteobacteria	Caulobacterales	Maricaulaceae	Photocaulis	sulfatitolerans	(11)
Proteobacteria	α -Proteobacteria	Rhodospirillales	Rhodospirillaceae	Aerophototrophica	crusticola	(10)
Proteobacteria	α -Proteobacteria	Hyphomicrobiales	Phyllobacteriaceae	Hoeflea	phototrophica	(3)
Proteobacteria	α -Proteobacteria	Hyphomicrobiales	Methylobacteriaceae	Methylorubrum	extorquens	(8)
Proteobacteria	β -Proteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans	planktonicus	(7)
Proteobacteria	β -Proteobacteria	Burkholderiales	Comamonadaceae	Roseateles	depolymerans	(2)
Proteobacteria	β -Proteobacteria	Rhodocyclales	Rhodocyclaceae	Aquincola	tertiaricarbonis	(5)
Proteobacteria	γ -Proteobacteria	Cellvibrionales	Haliaceae	Congregibacter	litoralis	(4)
Proteobacteria	γ -Proteobacteria	Cellvibrionales	Haliaceae	Chromocurvus	halotolerans	(4)
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	phototrophica	(5)
Acidobacteriota	"Acidobacteria"	Acidobacteriales	Acidobacteriaceae	Chloracidobacterium	thermophilum	(9)

(1) (Yurkov and Beatty, 1998); (2) (Rathgeber *et al.*, 2004); (3) (Yurkov and Csotonyi, 2009); (4) (Yurkov and Hughes, 2013); (5) (Koblížek, 2015); (6) (Koblížek, 2015); (7) (Kasalický *et al.*, 2018); (8) (Peyraud *et al.*, 2011); (9) (Tank and Bryant, 2015); (10) (Tang *et al.*, 2019); (11) (Kuzyk *et al.*, 2022).

However, AAP likely possess additional and potentially more significant roles than merely to carbon-cycling. Many species have extremely high levels of tolerance to metal(loid) oxides, including tellurite, tellurate, selenite, selenate, and vanadate (Csotonyi *et al.*, 2015; Maltman *et al.*, 2017; Sabaty *et al.*, 1998). Whereas tellurite is toxic to most other life at levels as low as 1 µg/mL, some AAP can tolerate over 2500 µg/mL (Yurkov *et al.*, 1996). In addition to their staggering tolerance mechanism, many can cytoplasmically reduce metal(loid) oxides to elemental forms, which are considerably less toxic in general. Pure crystals produced inside cytoplasmic space may have important application potential for bioremediation and bioreclamation (Maltman and Yurkov, 2018). As bacteria collect and neutralize the harmful oxides, they render the surrounding environment less toxic, allowing life to continue. Furthermore, the acquisition and reduction to elemental metals is favorable for bio-mining, where the cells could be harvested, and amassed valuable pure metals used for industrial purposes, such as the manufacture of semiconductors for electronics (Ilyas *et al.*, 2017).

But in addition to applied industrial potential, this capability is quite interesting biologically. If most AAP transform metal compounds quite readily, is it a mechanism that often functions in the environment as a key geobiochemical pathway they influence? Most strains tested for reduction originated from extreme environments, whereas AAP from mundane habitats such as lakes still await additional follow-up study to determine their behavior. Moreover, metal(loid) oxide reduction is only one possibility, and several other types of metal transformations could exist. *Chromocurvus halotolerans*, EG19^T has released a brown pigment into the growth medium, proposed to be a siderophore or an external metal chelating small molecule (Csotonyi *et al.*, 2011). Perhaps AAP may have several strategies of collecting and modifying metals, and it is their important feature and niche. As discussed above about AOP, Fe and Zn are scarcely dissolved in aquatic habitats, and the AAP potential to collect and transform them may be of critical importance, as both cations are essential for biological processes, especially photosynthesis.

Further to this, it is also unknown whether AAP act on other biogeochemical cycles with any significance, including nitrogen and phosphorus transformations. Physiologically and phylogenetically rhizobia-related AAP have been discovered, and they could potentially possess similar nitrogen fixing potential (Fleischman and Kramer, 1998). Moreover, massive accumulations of polyphosphates have been observed in a few AAP, which could be a possible phosphorus sink (Yurkov and Beatty, 1998). Each pathway requires thorough

investigation to see if and how AAP participate, as cyanobacteria are quite influential in many cycles, not just limited to carbon, therefore AAP may have additional important abilities as well.

Regarding cyanobacteria and their association with AAP, it is noteworthy that starting with the first ever isolated AAP (Harashima *et al.*, 1978), many species have been recovered from cyanobacterial or algal mats (Csotonyi *et al.*, 2010; Yurkov *et al.*, 1994), suggesting close relations and contacts. As both tolerate oxygen, and many phototrophs have been found regulated in microbial mats by O₂ presence as early as Winogradski's famous column discoveries (Dworkin and Gutnick, 2012), AAP and AOP may relate commensally, where AOP potentially share organic carbon and AAP may contribute to metal availability. Such ideas require factual experimental support, where the relationship between both types of phototrophs needs to be investigated, and the metal transformations of AAP needs to be determined, including from isolates of freshwater habitats.

Further functions and key roles of AAP have been discovered relating to the foodweb, particularly being preyed upon by filter feeders and grazers including snails *Physella johnsoni* (Bilyj *et al.*, 2014), zooplankton or protists (Garcia-Chaves *et al.*, 2015). As they make up a large part of the microbial community in many ecosystems, these bacteria are likely consumed by various predators, also warranting future investigation.

With all information described above in mind, AAP populations are important to consider when observing microbial communities in aquatic habitats. They have been at high numbers in many ecosystems, likely associated to other important members included AOP, and presumably feed many higher level organisms in the foodweb. Chl *a* measurement in Lake Winnipeg confidently predicted massive AOP presence, but no BChl *a* had yet been estimated, hence warranting further exploration. Finally, the linking of AAP counts to environmental parameters and other biological populations may also provide insights into the niche they occupy.

1.4. Thesis Objectives

Lake habitats are finite freshwater resources that still have many research knowledge gaps regarding their ecosystem dynamics, particularly in the area of microbial ecology. As Lake Winnipeg is one of the largest freshwater reservoirs in the world, its predicament of eutrophication and lack of bacteriological analysis, made it an exceptional environment to study. Furthermore, since AAP have been isolated and quantified from freshwater far less frequently when compared to marine or extreme environments, but still have much in the way of diversity, physiology, and ecology to discover, we proposed that: *“Aerobic anoxygenic phototrophs are an abundant, highly adaptable physiological group in Lake Winnipeg due to the eutrophic nature of the habitat. They play a key role in the carbon cycle, biogeochemical turnover of elements, and the food chain.”* To prove such a hypothesis, the following goals were implemented:

- **To determine how AAP fit into the microbial community of Lake Winnipeg, and understand the influence of environmental factors on different populations spatially and temporally.**
 - *Investigate how the invasion of zebra mussels, *D. polymorpha*, may affect the microbial community and AAP populations.*
- **Identify AAP from Lake Winnipeg to assess their physiology, taxonomy, and phylogeny. Compare and distinguish isolated strains to better explain diversity and niche.**
 - *Examine purified AAP to possibly find novel taxons and expand upon the knowledge of diversity of pigment production and photosynthesis. Potentially describe new taxa of phototrophs using polyphasic approach of bacterial systematics.*
- **Investigate AAP metal transformations to see if features of isolates from extreme habitats can be found among mundane Lake Winnipeg strains.**
 - *Identify biogeochemical functions of AAP by exploring additional metabolic pathways.*

1.5. Bibliography

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2. Chapter 2.

Seasonal dynamics of Lake Winnipeg's microbial communities reveal aerobic anoxygenic phototrophic populations coincide with sunlight availability

Steven B. Kuzyk, Xiao Ma and Vladimir Yurkov

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2.0. Preamble

Understanding the niche of AAP was the focus of our work. Analyzing these bacteria in a habitat at regular temporal and spatial intervals would allow for the comparison of bacterial numbers and diversity to measured environmental factors and the determination of what drives their population dynamics. Furthermore, prior to our work, Lake Winnipeg, the 11th largest freshwater lake in the world, was virtually unstudied for its microbial communities.

2.1. Contributions statement

Conceptualization, S.B.K. and V.Y.; methodology, S.B.K. and V.Y.; software, S.B.K.; validation, S.B.K. and V.Y.; formal analysis, S.B.K. and X.M.; investigation, S.B.K. and X.M.; resources, S.B.K. and V.Y.; data curation, S.B.K.; writing—original draft preparation, S.B.K.; writing, review and editing, S.B.K. and V.Y.; visualization, S.B.K. and V.Y.; supervision, V.Y.; project administration, V.Y.; funding acquisition, V.Y.

S.B.K. was the major contributor to the research. X.M. assisted in several littoral sampling trips and bacterial enumerations. V.Y. was the principal investigator.

2.2. Abstract

In this first comprehensive study of Lake Winnipeg's microbial communities, limnetic and littoral euphotic zones were examined during each season from 2016 through 2020. Classical cultivation and modern high-throughput sequencing techniques provided quantification and identification of key phototrophic populations, including aerobic anoxygenic phototrophs (AAP). Annual dynamics found total heterotrophs reached 4.23×10^6 CFU/g in littoral sands, and 7.69×10^4 CFU/mL in summer littoral waters on oligotrophic media, higher counts than for copiotrophic compositions. Limnetic numbers inversely dipped to 4.34×10^3 CFU/mL midsummer. Cultured AAP did not follow heterotrophic trends, instead peaking during the spring in both littoral and limnetic waters as 19.1 and 4.7% of total copiotrophs, or 3.9 and 4.9% of oligotrophs, decreasing till autumn each year. Complementary observations came from environmental 16S V4 rRNA gene analysis, as AAP made up 1.49 and 1.02% of the littoral and limnetic sequenced communities in the spring, declining with seasonal progression. Spatial and temporal fluctuations of microbes compared to environmental factors exposed photosynthetic populations to independently and regularly fluctuate in the ecosystem. Oxygenic phototrophic numbers expectantly matched the midsummer peak of Chl *a* and *b*, oxygenic photosynthesis related carbon fixation, and water temperature. Independently, AAP particularly colonized spring littoral areas more than limnetic, and directly corresponded to habitat conditions that specifically promoted growth: The requirement of light and organic material.

Keywords: Microbial ecology of lakes; Aerobic anoxygenic phototrophs; Bacterial community; Lake Winnipeg; Food web dynamics; Picoplankton; Bacterioplankton

2.3. Introduction

Lakes are the largest accumulations of liquid freshwater on Earth yet make up only 1% of the world's surface. These habitats have maintained incredible importance as sources for fisheries, drinking water and irrigation supply. The North American continent contains many of the highest volume natural water reservoirs, where the detrimental effects of anthropogenic eutrophication have caused the Laurentian Great Lake ecosystems to be rigorously monitored (Reed and Hicks, 2011; Mou *et al.*, 2013; Nakatsu *et al.*, 2019; Paver *et al.*, 2020). In contrast, the isolated northern Great Bear, Great Slave, and Lake Winnipeg have received less attention (Evans, 2000; Boguski, 2015; Koehl *et al.*, 2021). Of these three, Great Bear Lake has incurred the least human exposure, and Great Slave similarly maintained low land usage within its watershed. Inversely, Lake Winnipeg has been subjected to considerable nutrient loading due to its massive drainage basin spanning over 1,000,000 km² across every Canadian prairie province and some American states (Matisoff *et al.*, 2017). All significant agricultural, industrial, municipal, and urban runoff eventually enters its waters prior to draining into the Hudson's Bay of the Arctic Ocean via a single outlet, the Nelson River. In response, studies have predominantly focused on chemical and environmental fluctuations, as well as the impact to fish populations and other eukaryotes, presented thoroughly in recent collaborative efforts by academic researchers and provincial and federal governments (MWSD, 2011; MARD, 2020).

Nonetheless, some knowledge gaps have remained, including a lack of information regarding the primary producers, consumers, and recyclers of nutrients and biomass. The organisms at the base of food web dynamics, the microbial community, were thus far largely unexplored.

Algae and cyanobacteria have been commonly characterized in freshwaters, acting as aerobic oxygenic photoautotrophs (AOP) that use chlorophyll (Chl)-based photosynthesis, capable of fixing CO₂ into organic compounds consumable throughout the food chain (Kling *et al.*, 2011; Pick, 2016). However, as published for other great lakes (Newton *et al.*, 2011), AOP made up only a portion of the microbial community, where many other photoheterotrophs, photoautotrophs, chemoheterotrophs, and chemoautotrophs also inhabited and influenced the ecosystem. Phototrophic populations have been particularly diverse in aquatic habitats, with predominant groups differentiated by their photosynthetic systems. Anaerobic anoxygenic phototrophs (AnAnP) produce biological energy from light using bacteriochlorophyll (BChl) mediated photophosphorylation in the absence of oxygen, typical of stratified or benthic zones (Vila and Abella, 1994). Aerobic anoxygenic phototrophs (AAP) similarly use BChl *a* as a primary pigment, but require oxic euphotic

habitats rather than anoxic ones. Photosynthesis has likely evolved in AAP to act as an auxiliary energy production strategy to outcompete typical cohabitating heterotrophs by consuming organics faster due to the supplemental use of light energy (Yurkov and Beatty, 1998; Yurkov and Hughes, 2017). In addition to assorted photosynthesizing populations, numerous other trophic groups co-exist with harmonious and complex interactions occurring on the microscopic scale in each ecosystem (Vila *et al.*, 1998; Koblížek, 2015).

Regarding aquatic microbial ecology, many methods have been developed to study either individual populations or entire communities, uncovering a wide spectrum in microbial species composition among freshwater systems (Newton *et al.*, 2011). While variability could indeed be present, results have been difficult to compare due to dissimilar sampling methods/approaches. Furthermore, limited sampling locations and infrequent return visits have yet to address spatial and temporal system dynamics and stabilities (Reed and Hicks, 2011; Mou *et al.*, 2013; Nakatsu *et al.*, 2019; Paver *et al.*, 2020). To determine such ecosystem patterns, Lake Winnipeg's microbial communities were investigated at multiple euphotic zones, over all four seasons during the past 5 years. Chosen limnetic and littoral sites were analyzed with classical microbial cultivation and modern sequencing techniques in addition to physico-chemical parameter collection, a crucial combination which allowed for both diversity and functionality to be monitored in unison. Among the phototrophic groups, AAP were specifically regarded as they have been discovered in a vast array of environments at high numbers (Yurkov and Beatty, 1998; Koblížek, 2015; Bilyj *et al.*, 2014; Fauteux *et al.*, 2015). However, their role in ecology and biogeochemical cycling of elements has remained unclear. The abundance, fluctuations, and relation of AAP to other populations of the community were therefore highlighted in this work.

2.4. Materials and Methods

2.4.1. Sampling and environmental parameters

Lake Winnipeg was visited during the spring in May-June, summer between July-August, fall amid September-October, and winter among February-March, with 4 years' worth of seasons occurring from 2016 to 2020 (Table 2.1). Euphotic regions were sampled 0.25 m below the surface at 5 littoral areas accessed by land, and 5 limnetic zones retrieved using a rosette canister deployed off the research vessel M.V. *Namao* during open-water months (Fig. 2.1A), or from helicopter transport and ice coring equipment when the lake was frozen (Fig. 2.1B). Each littoral site was located where lake water was 0.5 m deep, light reached the bottom, and had sediment samples collected.

Table 2.1. Sampling dates and environmental parameters. Listed for each site (1-10).

Year	Season	Characteristic	1 Littoral	2 Littoral	3 Littoral	4 Littoral	5 Littoral	6 Limnetic	7 Limnetic	8 Limnetic	9 Limnetic	10 Limnetic
2016	Spring	Date	2016.06.14	2016.06.14	2016.06.14	2016.06.14	2016.06.14	2016.06.02	2016.06.02	2016.06.07	2016.06.07	2016.06.07
		Time	8:12	14:07	10:24	11:27	12:16	12:20	15:10	11:52	14:15	17:45
		Daylight (h)	16:26:57	16:24:45	16:26:16	16:27:42	16:26:48	16:15:22	16:16:51	16:24:32	16:18:15	16:25:47
		Water pH	8.3	8.6	8.3	8.6	8.5	8.1	8.5	8.1	8.2	7.8
		Water (°C)	14.0	13.0	15.0	11.0	18.0	12.0	13.0	14.2	15.1	14.6
		Air (°C)	26.0	28.0	27.0	27.0	24.0	10.3	12.0	10.5	15.0	12.6
		Water clarity (m)	0.81	0.50	0.20	0.30	0.76	0.80	1.10	1.25	1.05	0.8
		Light intensity (lux)	106000	125500	126300	124700	127400	78000	107800	126700	127000	33600
	Summer	Date	2016.08.25	2016.08.25	2016.08.25	2016.08.25	2016.08.25	2016.07.29	2016.07.29	2016.07.19	2016.07.19	2016.07.19
		Time	7:38	9:13	9:51	10:37	11:25	18:00	14:03	11:58	14:44	18:23
		Daylight (h)	13:54:19	13:53:29	13:54:03	13:54:36	13:54:16	15:20:21	15:27:29	15:54:59	15:56:00	15:56:04
		Water pH	8.5	8.4	8.6	8.8	8.8	8.4	8.3	8.0	7.8	8.1
		Water (°C)	23.0	25.0	23.0	22.0	22.0	22.7	22.1	20.9	21.1	21.1
		Air (°C)	13.0	17.0	18.5	18.0	19.0	28.5	24.0	23.2	23.5	23.5
		Water clarity (m)	0.20	0.10	0.20	0.20	0.10	0.50	0.75	0.40	0.30	0.4
		Light intensity (lux)	47300	106000	40500	46600	129100	120400	100400	50500	115300	102700
	Fall	Date	2016.10.17	2016.10.17	2016.10.17	2016.10.17	2016.10.17	2016.09.26	2016.09.26	2016.09.12	2016.09.12	2016.09.12
		Time	9:57	11:25	12:12	12:50	13:25	10:31	12:51	13:00	19:11	14:33
		Daylight (h)	10:35:32	10:36:13	10:35:45	10:35:19	10:35:35	11:46:46	11:58:12	12:48:05	12:48:16	12:48:17
		Water pH	8.0	8.3	8.3	8.3	8.5	8.0	8.2	8.3	8.3	8.2
		Water (°C)	9.0	8.0	9.0	13.0	14.0	14.9	16.1	18.9	14.6	18.9
		Air (°C)	13.0	11.0	12.5	13.0	15.0	10.7	12.5	13.5	12.0	15.5
		Water clarity (m)	0.10	0.20	0.20	0.20	0.13	0.35	0.40	0.25	0.45	0.5
		Light intensity (lux)	116800	117500	126600	127800	122800	22000	45000	30000	3050	32000
2017	Winter	Date	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2017.02.22	N/A	N/A
		Time	N/A	N/A	N/A	N/A	N/A	N/A	N/A	12:03	N/A	N/A
		Daylight (h)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10:31:04	N/A	N/A
		Water pH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7.8	N/A	N/A
		Water (°C)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.1	N/A	N/A
		Air (°C)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-1.4	N/A	N/A
		Water clarity (m)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.80	N/A	N/A
		Light intensity (lux)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Spring	Date	2017.05.08	2017.05.08	2017.05.08	2017.05.08	2017.05.08	2017.05.31	2017.05.31	2017.06.06	2017.06.06	2017.06.06
		Time	7:45	9:15	9:55	10:38	11:10	11:54	14:03	15:50	21:40	17:50
		Daylight (h)	15:10:10	15:08:42	15:09:43	15:10:40	15:10:04	16:11:24	16:12:52	16:22:54	16:24:03	16:24:09
		Water pH	8.7	8.4	8.4	8.2	8.7	8.4	8.4	8.5	8.4	8.3
		Water (°C)	16.0	19.5	17.5	17.0	17.5	11.7	12.7	15.1	13.2	16.2
		Air (°C)	22.0	18.0	20.5	24.5	22.0	18.0	13.6	20.7	20.6	19.6
		Water clarity (m)	0.03	0.38	0.13	0.13	0.05	1.40	1.40	1.50	1.40	1.5
		Light intensity (lux)	106100	114300	122800	119300	126100	127000	129000	125900	60000	123200
	Summer	Date	2017.08.04	2017.08.17	2017.08.17	2017.08.17	2017.08.17	2017.08.04	2017.08.04	2017.07.25	2017.07.25	2017.07.25
		Time	21:00	8:48	9:45	10:25	11:06	10:46	12:45	12:06	16:27	14:29
		Daylight (h)	15:07:10	14:22:39	14:23:23	14:24:05	14:23:39	15:08:38	15:09:40	16:19:44	15:40:51	15:40:55
		Water pH	8.3	9.1	8.8	8.9	9.2	8.2	8.3	8.2	8.3	8.1
		Water (°C)	22.5	22.0	24.0	22.0	22.5	22.0	22.9	20.8	22.6	21.3
		Air (°C)	22.2	14.0	18.0	18.5	19.0	20.3	22.1	21.8	21.8	22.6
		Water clarity (m)	0.90	1.30	1.17	1.04	>1.32	0.45	0.70	0.35	0.25	0.4
		Light intensity (lux)	N/A	74400	109700	110100	120400	128700	143100	129500	11340	56200
	Fall	Date	2017.10.01	2017.10.01	2017.10.15	2017.10.15	2017.10.15	2017.10.04	2017.10.04	2017.09.18	2017.09.18	2017.09.18
		Time	8:23	10:21	7:30	8:10	8:50	13:35	13:47	18:20	23:10	21:20
		Daylight (h)	11:36:25	11:36:38	11:36:29	11:36:21	11:36:26	11:24:47	11:24:34	12:26:01	12:26:06	12:26:07
		Water pH	8.8	9.1	8.0	8.2	8.1	8.2	8.2	8.5	8.0	8.4
		Water (°C)	9.5	13.0	16.0	15.0	15.0	13.1	12.3	15.5	13.0	15.4
		Air (°C)	14.0	17.0	4.0	5.0	5.5	13.4	11.3	11.9	15.5	14.8
		Water clarity (m)	0.84	0.48	0.90	1.00	0.35	0.90	0.90	0.79	1.07	0.79
		Light intensity (lux)	43700	61000	2020	16240	25220	21700	41800	125400	72900	119300
2018	Winter	Date	N/A	N/A	N/A	N/A	N/A	2018.02.26	N/A	2018.02.26	N/A	N/A
		Time	N/A	N/A	N/A	N/A	N/A	15:30	N/A	10:35	N/A	N/A
		Daylight (h)	N/A	N/A	N/A	N/A	N/A	10:45:52	N/A	10:45:22	N/A	N/A
		Water pH	N/A	N/A	N/A	N/A	N/A	7.71	N/A	7.72	N/A	N/A
		Water (°C)	N/A	N/A	N/A	N/A	N/A	-0.1	N/A	-0.1	N/A	N/A
		Air (°C)	N/A	N/A	N/A	N/A	N/A	-5.0	N/A	-2.0	N/A	N/A
		Water clarity (m)	N/A	N/A	N/A	N/A	N/A	1.00	N/A	1.40	N/A	N/A
		Light intensity (lux)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 2.1. (continued)

Year	Season	Characteristic	1	2	3	4	5	6	7	8	9	10
			Littoral	Littoral	Littoral	Littoral	Littoral	Limnetic	Limnetic	Limnetic	Limnetic	Limnetic
2018	Spring	Date	2018.05.29	2018.05.29	2018.05.29	2018.05.29	2018.05.29	2018.06.13	2018.06.12	2018.06.13	2018.06.04	2018.06.04
		Time	7:42	9:19	10:07	10:52	11:35	9:26	10:00	12:53	8:59	10:14
		Daylight (h)	16:05:02	16:03:03	16:04:25	16:05:42	16:04:54	16:23:20	16:22:17	16:22:34	16:20:45	16:30:35
		Water pH	9.2	9.4	9.1	9.3	8.7	8.2	8.1	8.2	8.0	8.2
		Water (°C)	15.0	19.0	15.5	16.0	20.0	16.2	13.7	12.9	17.2	15.6
		Air (°C)	20.0	25.5	27.5	27.0	26.0	16.5	16.0	18.5	20.3	20
		Water clarity (m)	1.07	0.05	0.52	1.00	0.97	1.96	1.20	2.33	2.33	2.33
		Light intensity (lux)	60400	118800	123500	124900	124800	99000	13700	92100	119400	124200
	Summer	Date	2018.07.30	2018.08.08	2018.08.08	2018.08.08	2018.08.08	2018.08.07	2018.07.31	2018.08.07	2018.07.30	2018.07.30
		Time	3:50	8:07	8:51	9:36	10:17	10:35	9:44	12:00	12:08	10:39
		Daylight (h)	15:23:11	14:53:45	14:54:41	14:55:33	14:55:00	14:59:45	15:22:56	15:00:54	15:01:39	15:01:43
		Water pH	8.5	8.8	8.4	8.6	8.8	8.3	8.2	8.3	8.1	8.2
		Water (°C)	21.5	24.0	22.0	22.0	21.5	20.0	21.2	20.2	21.1	20.6
		Air (°C)	31.0	22.0	24.0	24.5	24.0	19.1	18.3	18.9	15.8	25.6
		Water clarity (m)	0.81	0.42	0.50	0.58	0.20	0.39	0.84	0.67	0.56	0.53
		Light intensity (lux)	105300	61500	82900	86900	104700	1170	2340	9850	3240	8320
	Fall	Date	2018.10.22	2018.10.08	2018.10.08	2018.10.08	2018.10.08	2018.10.22	-	2018.09.25	-	2018.09.25
		Time	18:00	8:34	9:20	10:15	10:50	10:19	-	11:32	-	13:53
		Daylight (h)	10:18:59	11:11:19	11:11:03	11:10:48	11:10:57	10:18:11	Ship	12:00:03	Ship	12:00:02
		Water pH	8.0	8.5	8.3	8.3	8.3	8.2	Repaired	8.3	Repaired	8.3
		Water (°C)	3.0	4.5	6.0	6.0	6.5	4.1	No	12.1	No	12.2
		Air (°C)	1.5	4.0	4.5	4.5	6.0	2.6	Sampling	7.2	Sampling	9.3
		Water clarity (m)	0.25	0.15	0.41	0.67	0.29	0.65	-	0.50	-	0.86
		Light intensity (lux)	6890	4800	11240	14090	2250	12270	-	128700	-	122800
2019	Winter	Alternative Sites	N/A	N/A	N/A	N/A	N/A	W10	W1	W9	W6	W8
		Date	N/A	N/A	N/A	N/A	N/A	2019.02.28	2019.02.20	2019.02.28	2019.02.21	2019.02.28
		Time	N/A	N/A	N/A	N/A	N/A	13:20	9:00	9:30	10:50	11:30
		Daylight (h)	N/A	N/A	N/A	N/A	N/A	10:41:11	10:12:29	10:40:37	10:19:28	10:50:10
		Water pH	N/A	N/A	N/A	N/A	N/A	7.8	8.17	7.7	8.09	7.96
		Water (°C)	N/A	N/A	N/A	N/A	N/A	-0.5	-1.0	-0.7	-1.0	0.8
		Air (°C)	N/A	N/A	N/A	N/A	N/A	-8.0	-18.9	-9.0	-17.1	-10
		Water clarity (m)	N/A	N/A	N/A	N/A	N/A	1.8	0.70	1.2	0.60	0.6
		Light intensity (lux)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Spring	Sites	1	2	3	4	5	6	7	8	9	10
		Date	2019.06.03	2019.06.08	2019.06.08	2019.06.05	2019.06.08	2019.06.03	2019.06.03	2019.06.05	2019.06.05	2019.06.05
		Time	7:07	7:38	8:28	7:00	9:26	11:30	2:30	10:25	16:45	13:12
		Daylight (h)	16:13:44	16:18:44	16:20:13	16:17:33	16:20:44	16:15:44	16:17:12	16:20:50	16:21:58	16:22:05
		Water pH	8.4	7.5	8.1	8.0	7.6	8.2	8.4	8.5	8.0	8.5
		Water (°C)	13.0	16.0	13.0	12.0	15.5	10.5	11.1	11.2	12.4	12
		Air (°C)	12.9	18.0	18.0	12.1	17.0	10.2	11.3	12.9	18.7	17.1
		Water clarity (m)	0.76	0.38	0.34	0.53	>1.50	0.9	0.8	1.2	1.6	1.1
		Light intensity (lux)	12020	8550	8140	106800	28700	6630	37800	126200	48400	66200
	Summer	Date	2019.08.06	2019.08.13	2019.08.13	2019.08.13	2019.08.13	2019.08.06	2019.08.06	2019.07.23	2019.07.23	2019.07.23
		Time	7:20	7:02	8:20	10:20	9:05	11:33	2:15	11:24	17:15	14:05
		Daylight (h)	15:02:21	14:37:59	14:38:49	14:39:36	14:39:07	15:03:42	15:04:42	15:46:41	15:47:39	15:47:44
		Water pH	8.4	8.7	8.4	8.3	8.2	8.4	8.3	8.0	8.1	8
		Water (°C)	26.0	25.0	23.0	24.0	19.0	22.2	22.1	21.8	21.3	22.7
		Air (°C)	16.7	15.5	25.5	24.0	23.0	21.8	24.8	23.1	23.2	24.1
		Water clarity (m)	0.65	0.39	0.61	0.51	1.02	0.50	1.40	0.60	0.85	0.8
		Light intensity (lux)	15310	6030	95500	118000	116500	41500	64600	119700	122400	117800
	Fall	Date	2019.10.02	2019.09.22	2019.09.22	2019.09.22	2019.09.22	2019.10.02	2019.10.02	2019.09.16	2019.09.16	2019.09.16
		Time	8:55	8:11	11:03	12:28	11:55	13:10	16:10	13:03	19:00	15:45
		Daylight (h)	11:34:29	12:12:21	12:12:24	12:12:26	12:12:25	13:28:13	11:34:07	12:35:34	12:35:42	12:35:42
		Water pH	8.2	8.2	8.1	8.0	8.2	8.2	8.2	8.2	8.0	8.2
		Water (°C)	9.0	15.0	15.5	14.0	16.0	11.9	10.4	15.8	16.8	16.2
		Air (°C)	3.1	14.5	16.0	17.0	14.5	4.7	5.2	21.4	23.1	27.7
		Water clarity (m)	0.33	0.17	0.27	0.19	0.13	0.50	0.70	0.50	0.09	0.85
		Light intensity (lux)	4380	10980	122000	133500	192000	29400	18800	35200	N/A	7750
2020	Winter	Alternative Sites	N/A	N/A	N/A	N/A	N/A	W10	W1	W9	W6	W8
		Date	N/A	N/A	N/A	N/A	N/A	2020.02.24	2020.03.04	2020.02.24	2020.03.03	2020.02.24
		Time	N/A	N/A	N/A	N/A	N/A	14:10	9:30	13:15	9:30	10:30
		Daylight (h)	N/A	N/A	N/A	N/A	N/A	10:36:30	11:05:44	10:35:53	11:03:09	10:33:30
		Water pH	N/A	N/A	N/A	N/A	N/A	6.3	6.66	7.1	6.53	6.34
		Water (°C)	N/A	N/A	N/A	N/A	N/A	0.4	-9.2	0.3	-10.1	-2.9
		Air (°C)	N/A	N/A	N/A	N/A	N/A	-0.9	-0.8	-0.5	-0.7	-0.6
		Water clarity (m)	N/A	N/A	N/A	N/A	N/A	1.00	0.70	0.70	0.50	0.6
		Light intensity (lux)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

N/A, Not analyzed

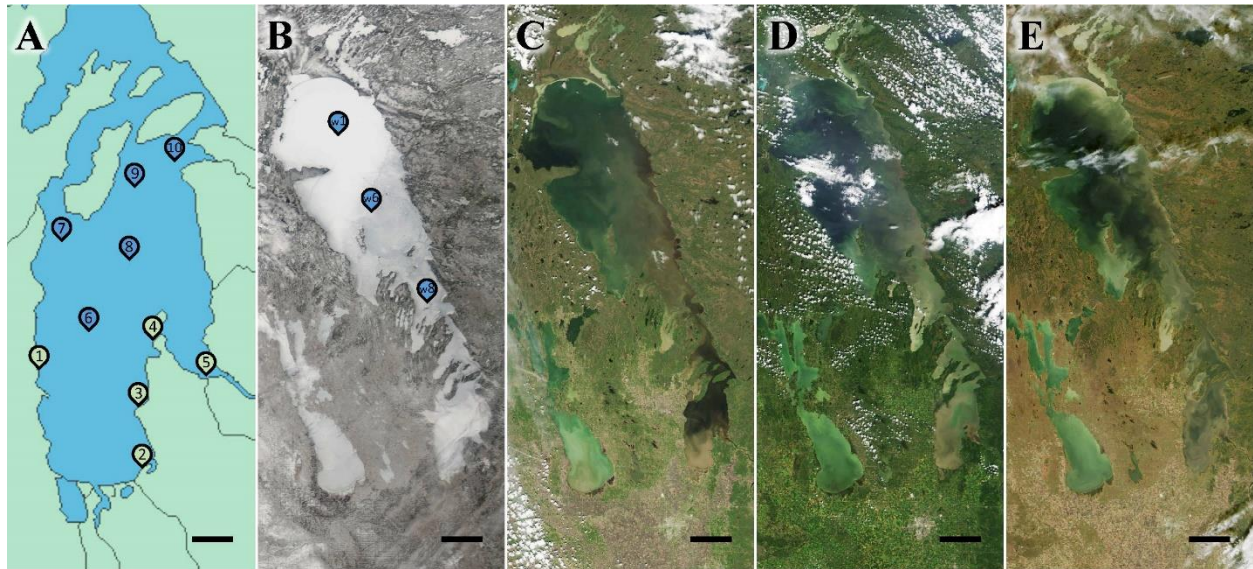


Fig. 2.1. Lake Winnipeg seasonal progression and sampling locations. (A) Map of the South basin of Lake Winnipeg, with littoral (green) and limnetic (blue) sites selected for study: S1-5, S6-10 (W1, W6 and W8 alternatively), respectively; Scale bar, 10 km. Satellite images of entire waterbody depicting seasonal change to lake habitat and surrounding lands during (B) Winter, March 26nd 2017 indicating additional under-ice sites, (C) Spring, May 31st 2017, (D) Summer, August 3rd 2017, and (E) Fall, October 11th 2017; Scale bars, 40 km. Photographs provided by Dr. K. Scott and the Lake Winnipeg Research Consortium (LWRC), in partnership with the Rapid Response Imagery from the Land Atmosphere Near-real time Capability for EOS (LANCE) system operated by the NASA/GSFC/Earth Science Data and Information System (ESDIS).

Littoral areas included Site 1 at Gimli Beach (50°38'2"N, 96°59'2"W); Site 2, Patricia Beach (50°25'25"N, 96°37'2"W); Site 3, Grand Beach (50°34'11"N 96°35'59"W); Site 4, Victoria Beach (50°42'16"N, 96°33'49"W); and Site 5 near the Sagkeeng First Nation Community (50°37'11"N, 96°18'55"W). Limnetic locations were spread across the South Basin, chosen from the expansive list that the M.V. *Namao* frequently visited, denoted here as Sites 6–10, with the catalog names provided in quotations. Site 6 “W10” (50°50.799'N, 96°46.157'W), Site 7 “57B” (50°58.529'N, 96°52.551'W), Site 8 “W9” (51°01.334'N, 96°35.038'W), Site 9 “12B” (51°08.014'N, 96°37.159'W), and Site 10 “46S” (51°08.512'N, 96°25.27'W) were all kilometers apart from each other as well as from the nearest shoreline. Only Sites 6 and 8 could be sampled in the winter, and during 2019–2020, 3 additional sites were included, W1 (53°22'34.7880"N, -98°23'25.188"W), W6 (52°38'34.8"N, -97°44'5.1"W), and W8 (51°45'47.4474"N, -96°50'21.6954"W). While Sites 1–4, 6, and 7 were primarily fed by the waters of the Red River, Site 5 was downstream of the Winnipeg River, and 8–10 likely contained a mixture of the two inflows. Since Lake Winnipeg predominantly had a soft lakebed (Depew *et al.*, 2021), sandy-silty shorelines were common.

Water temperature and pH were recorded with a calibrated Beckman Φ 255 pH/Temp/mV meter, clarity by Secchi disk test, and light intensity (Lux) using a VWR Light Meter 21800-014. Data for daily average ambient temperatures, cloud coverage, and hours of sunlight were received from government sources. Major elemental compositions of limnetic water were determined via collision/reaction cell-based inductively coupled plasma mass spectrometry (CRC ICPMS). All 43 elements and their detection limits were collected by the Water Quality Management Section of Manitoba Environment, Climate and Parks Department, Canada and averages per season listed in Table S8.2. Concentrations of Chl *a*, Chl *b*, and BChl *a* from microbial communities were quantified as follows: A defined volume of liquid sample from each site was filtered through a 0.22 μ m pore size 47 mm nitrocellulose membrane (Millipore-Sigma, Burlington, MA, USA) to concentrate cells, prior to the addition of 7:2 acetone:methanol (5 mL) for the extraction of pigments overnight in the dark at 4°C. A Hitachi U-2010 spectrophotometer recorded absorption spectra from 350 to 1100 nm using a 50 mm path length quartz cuvette. Peaks at 663, 645, and 770 nm were used to calculate the concentration of Chl *a*, Chl *b*, and BChl *a*, respectively, per volume of water sample (Ritchie, 2018), based on classical approaches (Gregersen *et al.*, 2009; SCOR-UNESCO, 1966).

Table 2.2. Average elemental composition of limnetic site samples. Listed as ppm.

	2016			2017				2018
	Spring	Summer	Fall	Winter	Spring	Summer	Fall	Winter
Ag	L0.00001	0.000005	L0.00001	L0.00001	L0.00001	L0.00001	L0.00001	L0.00001
Al	0.191	1.5035	1.42	0.213	0.113	0.68	0.261	0.12
As	0.00188	0.001825	0.002395	0.00116	0.001645	0.00207	0.002885	0.001135
Ba	0.02625	0.0314	0.0371	0.00996	0.0229	0.0327	0.03275	0.0117
Be	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0001	L0.0001	L0.0001
Bi	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.00005	L0.00005	L0.00005
B	0.0315	0.0245	0.0305	L0.01	0.0245	0.033	0.038	0.01
Cd	0.00001	0.00002	0.00002	0.00001	L0.00001	0.00002	0.00000955	0.0000179
Ca	33.65	25.4	27.85	11.5	28.2	32	36.1	15.25
C (organic)	10.175	8.775	10.35	12.24	10.175	9.15	11.85	12.6
C (inorganic)	17.575	20.025	20.05	9.26	19.275	22	20.775	10.5
C (total)	27.75	28.8	30.4	21.5	29.45	31.15	32.625	23.1
Cs	L0.0001	0.000205	0.00018	L0.0001	L0.0001	0.000099	0.0000445	0.0000945
Cl	12.1	8.83	11.375	1.66	9.58	9.72	11.71	2
Cr	0.00034	0.002155	0.002055	0.00046	0.00023	0.001105	0.00048	0.00031
Co	L0.0002	0.0005	0.000505	L0.0002	L0.0002	0.00038	0.000175	L0.0001
Cu	0.001845	0.002465	0.002695	0.00134	0.001705	0.002355	0.00201	0.001565
Fe	0.1725	1.233	1.163	0.21	0.1025	0.7435	0.2525	0.1155
K	3.795	3.105	3.66	0.931	3.06	3.585	3.875	0.988
Li	0.01545	0.01125	0.0151	L0.002	0.01265	0.01585	0.0194	0.00365
Mg	15.3	11.655	14.32	3.66	14.05	15.65	18.2	5.17
Mn	0.01865	0.0561	0.0511	0.00672	0.02256	0.06615	0.0204	0.01021
Hg	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mo	0.000895	0.00069	0.00086	L0.0002	0.0007	0.00079	0.001058	0.0002075
Ni	0.00168	0.00275	0.00287	0.00084	0.001415	0.00256	0.00174	0.000885
N	0.8575	0.8425	0.8025	0.59	0.5875	0.765	0.7425	0.505
N (Kjeldahl)	0.6925	0.625	0.5775	0.41	0.5875	0.6125	0.6875	0.385
NH₃	0.01375	0.0105	0.01375	0.04	0.01025	0.0465	0.02125	L0.01
NO₂+NO₃	0.1655	0.218	0.22425	0.181	N/A	0.1515	0.0795	0.1215
Na	16.15	11.82	14.505	2.37	13.55	15.15	17.8	3.055
O	10.05	8.05	8.8	10.6	10.2	7.25	9.5	14.25
P	0.065	0.09325	0.13625	0.032	0.05575	0.11075	0.104	0.032
P (dissolved)	0.0395	0.0595	0.08425	0.024	0.0415	0.07475	0.07925	0.0235
P (inorganic)	0.054	0.0825	0.12425	0.028	0.0455	0.10575	0.098	0.03
P (particulate)	0.0265	0.041	0.054	L0.014	0.016	0.042	0.03	L0.014
P (reactive)	0.0405	0.06425	0.0995	0.026	0.03675	0.09075	0.0965	0.024
P (acid hydrolizable)	0.01625	0.0215	0.02675	L0.014	L0.014	0.01525	L0.014	L0.014
Pb	0.000115	0.000585	0.000555	0.00013	0.0001	0.000473	0.0001725	0.0001115
Rb	0.001765	0.004075	0.0042	0.0016	0.001555	0.00289	0.002265	0.00164
Sb	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	0.000195	0.00017	0.00012
Se	0.00025	0.00023	0.000275	L0.0001	0.000225	0.0002685	0.0003	0.0001595
Si	1.08	6.51	7.405	2.36	0.595	4.765	4.93	2.1
Sr	0.10195	0.0768	0.09675	0.0233	0.0866	0.1009	0.119	0.02915
Sn	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0001	L0.0001	L0.0001
SO₄	50.8	37.8	47.9	3.74	44.7	52	67.25	3.015
Te	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002
Tl	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	0.000015	L0.00001	L0.00001
Th	L0.0001	0.00036	0.00031	L0.0001	L0.0001	0.000155	L0.0001	L0.0001
Ti	0.00578	0.0538	0.0487	0.00661	0.00328	0.02135	0.007475	0.0035
U	0.0015	0.001085	0.00127	0.00011	0.001365	0.001535	0.00177	0.0001115
V	0.00189	0.005085	0.005545	0.00077	0.001475	0.00346	0.002965	0.000785
Zn	0.0014	0.0048	0.0053	0.0038	L0.002	0.004	L0.003	0.00355
Zr	L0.0004	0.00167	0.00149	L0.0004	L0.0004	0.000707	0.000336	0.0001625

Table 2.2. Continued. Listed as ppm.

	2018			2019				2020
	Spring	Summer	Fall	Winter	Spring	Summer	Fall	Winter
Ag	L0.00001	L0.00001	L0.00001	L0.00001	L0.00001	L0.00001	L0.00001	L0.00001
Al	0.1485	0.383	0.274	0.062	0.1109	0.27125	0.55075	0.151
As	0.001745	0.002185	0.00301	0.001145	0.002095	0.0024725	0.00307	0.00102
Ba	0.02315	0.02885	0.03235	0.01185	0.0224	0.026625	0.0351	0.01265
Be	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001
Bi	L0.00005	L0.00005	L0.00005	L0.00005	L0.00005	L0.00005	L0.00005	L0.00005
B	0.0275	0.026	0.035	L0.01	0.0195	0.02725	0.0355	L0.01
Cd	0.00001285	0.00001435	0.00001295	0.00003265	0.00000975	0.0000084	0.0000139	0.0000368
Ca	26.8	28.55	31	16.85	27.45	28.9	30.675	13.95
C (organic)	9.825	8.5	8.7	9.565	8.788333333	9.8025	9.1225	10.925
C (inorganic)	18.95	18.35	19.3	11.155	17.7	19.9	20.5875	9.45
C (total)	28.775	26.85	28	20.7	26.46666667	29.65	29.725	20.35
Cs	0.000036	0.0000615	0.0000465	0.0425	0.02775	0.05325	0.08075	0.1585
Cl	10.75	11.2	12.55	2.72	10.2025	10.8	9.7725	2.435
Cr	0.00032	0.00071	0.000875	0.000215	0.0002725	0.0005825	0.0009375	0.000475
Co	0.000125	0.00024	0.000195	L0.1	0.135	0.2075	0.285	L0.1
Cu	0.00175	0.002145	0.00212	0.00137	0.00186	0.0019025	0.00234	0.00158
Fe	0.1685	0.45	0.326	0.062	0.135	0.3175	0.47975	0.2285
K	2.98	3.6	3.795	1.067	3.8225	3.985	4.0925	1.106
Li	0.01295	0.01315	0.01775	0.00231	0.0127	0.013825	0.01585	0.002055
Mg	14.2	15.95	17.1	5.54	13.9	14.175	15.4	4.705
Mn	0.02705	0.0337	0.02735	0.003105	0.0192	0.0422	0.024825	0.006015
Hg	N/A	N/A	N/A	L0.000005	0.000925	0.0011875	0.00126125	L0.000005
Mo	0.0007175	0.0008535	0.0010595	0.000222	0.000791	0.0009255	0.00100125	0.0002005
Ni	0.00156	0.002135	0.00184	0.00077	0.0019475	0.00207	0.0020675	0.00096
N	0.44	0.5725	0.696666667	0.67	0.506666667	0.6175	0.84125	0.585
N (Kjeldahl)	0.4275	0.46	0.656666667	0.515	0.495	0.5825	0.7525	0.5
NH ₃	0.011	0.0185	0.015666667	L0.01	L0.01	0.01625	0.036375	L0.01
NO ₂ +NO ₃	0.0197	0.111075	0.0423	0.1555	0.01105	0.0366	0.091125	0.08355
Na	14.8	15.4	17.45	3.475	13.75	13.9	13.725	3.205
O	10.5	8.45	11.1	14.4	9.75	7.3	9.5	16.3
P	0.0569	0.093925	0.111733333	0.02545	0.064783333	0.10085	0.135375	0.0232
P (dissolved)	0.0359	0.06775	0.088533333	0.02	0.04735	0.074375	0.105425	0.0164
P (inorganic)	0.04665	0.0807	0.1012	0.0184	0.06505	0.0837625	0.120125	0.0181
P (particulate)	0.018	0.02905	0.0276	0.00545	0.019075	0.031125	0.047275	0.00675
P (reactive)	0.030475	0.0617	0.082966667	0.01485	0.03215	0.0574	0.103775	0.01375
P (acid hydrolyzable)	0.01615	0.019	0.0184	L0.0042	0.0329	0.026375	0.017525	0.00765
Pb	0.0001105	0.000227	0.000278	0.000074	0.000101	0.00017375	0.00031525	0.0001955
Rb	0.001705	0.00244	0.00228	0.00159	0.00155	0.00207	0.002655	0.001755
Sb	0.00011	0.00013	0.000185	0.00026	0.00013	0.00014	0.0001775	0.0001
Se	0.000234	0.000244	0.0002945	0.0001185	0.00021275	0.00022725	0.0002935	0.0001215
Si	2.27	4.565	5.34	2.585	0.8175	3.3125	7.525	2.05
Sr	0.089	0.0927	0.111	0.03275	0.088475	0.0966	0.10735	0.03075
Sn	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	0.0001325	0.00013
SO ₄	46.45	51.15	60.5	4.86	43.225	52.25	56.5	4.615
Te	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002	L0.0002
Tl	L0.00001	0.000013	0.000011	L0.00001	L0.00001	0.00001	0.000014	L0.00001
Th	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	L0.0001	0.0001425	L0.0001
Ti	0.00464	0.01244	0.008565	0.00143	0.0035525	0.0083225	0.018635	0.004565
U	0.001125	0.001085	0.00141	0.0001365	0.00105725	0.001355	0.00146	0.0001385
V	0.001575	0.002815	0.00299	0.00064	0.001995	0.0025025	0.004325	0.00075
Zn	L0.003	L0.003	0.00335	0.0067	L0.003	L0.003	0.005925	0.0091
Zr	0.000222	0.0003375	0.0003395	0.000123	0.000188	0.0002975	0.000795	0.000205

L, Less than the detection limit listed

2.4.2. Primary productivity assay

Community photo- and chemosynthetic carbon fixation was determined during the summer and fall of 2017, as well as the spring, summer, and fall of 2018 and 2019 by a ^{14}C labelled NaHCO_3 accumulation assay (Steemann-Nielsen, 1952), with some minor procedural modifications (Bilyj *et al.*, 2014). Winter in situ analysis was not possible due to the extreme cold temperatures, where shallow littoral areas were frozen solid, and limnetic zones were not accessible twice during the same day. Measurement involved a 3 to 24 h in situ incubation of 3 sealed glass Balch vials each containing 9 mL of natural lake water and 5 μCi ^{14}C -labelled NaHCO_3 . The first of three translucent vials contained only the two components, while the second was additionally wrapped in aluminum foil. The third uncovered vial contained the two components listed as well as 7 μmol diuron (Millipore-Sigma, Burlington, MA, USA), a photosystem II (PSII) inhibitor that prevents oxygenic photosynthesis without affecting anoxygenic photosynthesis (Bilyj *et al.*, 2014; Piwosz *et al.*, 2020). After residing in the lake, 400 μL of 40% formaldehyde (final as $\sim 2\%$) was added, stopping reactions and “fixing” them for storage. A 0.1 mL aliquot was later filtered through a 0.22 μm pore size filter (Millipore-Sigma, Burlington, MA, USA) until dry, prior to washing off unfixed ^{14}C with 5 mL of distilled water. Filters were placed in 0.5 mL ScintiSafe Plus 50% (Fisher Scientific, Waltham, MA, USA) scintillation fluid and measured for 5 min in a Beckman LS 6500. Counts were compared to 0.1 mL of unfiltered liquid for each sample. Carbonates and bicarbonates were determined by titration via a total alkalinity test (Franson, 1998). Rates of carbon fixation were calculated and equalized to a 24 h incubation using the equation:

$$Ca = \frac{r \cdot C_{in\ water}}{R \cdot t}$$

Here, Ca was the amount of fixed carbon (mg/L); $C_{in\ water}$, the total carbon present in water; R , the counts in control (unfiltered liquid sample); r , was counts in filtered material; and t , time of incubation. Chemosynthesis, anoxygenic or oxygenic photosynthesis related carbon fixation were calculated as the rates of vial 2, vial 3 minus 2, or vial 1 minus 3, respectively (Bilyj *et al.*, 2014).

2.4.3. Cultivation and enumeration of targeted populations

Water and sediment samples were decimally diluted to 10^{-7} and 0.1 mL aliquots were spread in triplicates on 2% agar plates. An assortment of media was designed (Table 2.3) to isolate and enumerate several specific physiological groups of microorganisms, with a main focus on phototrophs. Heterotrophs, including photoheterotrophic AAP, were cultivated with a standard rich organic (RO) medium (Yurkov and van

Table 2.3. Media composition used to cultivate different groups of microorganisms. Values are listed as g/L unless otherwise noted. All were adjusted to pH 5.9 prior to autoclaving.

Component	RO	OM	BG-11	PNSM	PM	DM
MgCl ₂	-	-	-	0.5	-	-
MgSO ₄	0.50	0.50	0.0750	-	-	0.50
KH ₂ PO ₄	0.30	0.30	0.0300	0.3	-	0.30
NH ₄ Cl	0.30	0.30	-	0.3	-	0.30
NaNO ₃	-	-	0.1500	-	-	-
KCl	0.30	0.30	-	-	-	-
CaCl ₂	0.05	0.05	-	0.1	-	0.10
Na ₂ CO ₃	-	-	0.0200	-	-	-
NaAcetate	1.00	0.10	-	1.0	0.50	-
NaCitrate	-	-	0.0060	-	-	-
Malic Acid	-	-	-	0.3	-	-
Na ₂ CO ₃	-	-	0.0200	-	-	-
Na ₂ -EDTA	-	-	0.0001	-	-	-
Ferric Ammonium Citrate	-	-	0.0060	-	-	-
Potato Extract*	-	-	-	-	*	-
Yeast Extract	1.00	0.05	-	0.2	1.00	-
Bacto Peptone	0.50	-	-	-	-	-
Casamino Acids	0.50	0.05	-	-	-	-
Trace Elements	2 mL	2 mL	2 mL	2 mL	2 mL	-
Vitamins	2 mL	2 mL	2 mL	2 mL	2 mL	-
<i>Added after autoclaving:</i>						
Cystine (0.3mM)	-	-	-	5 mL	-	-
Methionine (0.3mM)	-	-	-	5 mL	-	-
ph adjustments	A	A	B	C	B	D

A, 4.0uL/mL 0.5N NaOH added after autoclaving to achieve 7.5 pH

B, Self-adjusts to pH 7.5 during autoclaving process

C, 3.0uL/mL 0.5N NaOH added after autoclaving to achieve 7.5 pH

D, 2.4uL/mL 0.5N NaOH added after autoclaving to achieve 7.5 pH

* 1L of water per kg of peeled potatoes (organic better), cut in halves or quarters depending on size, enough water to cover all by 1-2 cm. Boiled for 1 h, strained water through cotton and dilute to twice volume.

Trace Elements	g/L		Essential Vitamins	g/L
FeSO ₄	0.300		Nicotinic Acid	0.40
MnCl ₂	0.003		Thiamin	0.40
CoCl ₂	0.005		Biotin	0.08
CuCl ₂	0.001		B ₁₂	0.02
NiCl ₂	0.002			
Na ₂ MoO ₄	0.003		Filter sterilize solution and refrigerate	
ZnSO ₄	0.005			
H ₃ BO ₃	0.002			
In 0.5L Adjust to 3.0pH				
Fill to 1L and refrigerate				

Gemerden, 1993). Oligotrophs were selected on an organic-limited medium (OM), which had all compounds as RO, with only 10% of the carbon sources. A comparable R2A composition has been successful for the cultivation of some AAP (Béjà *et al.*, 2002), where recent works have diluted its contents to improve isolation attempts (Zervas *et al.*, 2019; Kopejtká *et al.*, 2021). With such considerations, RO was implemented as a copiotrophic carbon source, OM as oligotrophic, whereas R2A was an intermediate with carbon content between RO and OM. Potato-broth based medium (PM) was included as it usually stimulated intense cellular pigmentation as a very rich nutrient source (Yurkov and Beatty, 1998), and helped to discover rare colony colorations. Purple non-sulfur bacteria and other AnAnP were enriched on a specific medium, PNSM, while AOP were selected on BG-11 (Bilyj *et al.*, 2014). Heterotrophs capable of tolerating toxic metalloids were counted on RO supplemented with 100 µg/mL K_2TeO_3 (RO_T), as some AAP have been found tolerant to high levels (Hughes *et al.*, 2017; Maltman and Yurkov, 2015). Media were autoclaved at pH 5.9 and adjusted to 7.5 before agar solidification. Cultures were incubated for 7 days at 28°C on RO, PM, R2A, and RO_T in the dark, and BG-11 and PNSM, under constant illumination. All were grown aerobically, except cultures on PNSM which developed in translucent anoxic Gas-Pak chambers.

Pigmented and total (including pigmented and colorless) colony forming units (CFU) were counted as CFU/g of sediments, and CFU/mL in water. To determine numbers of AAP, numerous colored colonies were re-plated onto medium of isolation to verify growth and pigmentation, except colonies from RO_T, which were instead passaged onto RO. Patched strains were later suspended in 0.2 mM TRIS-HCl buffer, pH 7.8 containing 70% glycerol to measure absorption spectra from 350 to 1100 nm using a HITACHI U2010 spectrophotometer. Strict aerobes containing BChl a bound to reaction center and light harvesting complexes were identified as AAP (Yurkov and Beatty, 1998). The numerical presence of AAP among pigmented heterotrophs was established (Table 2.4), and used to estimate their proportion among the total cultured copiotrophic or oligotrophic heterotrophic bacteria, on RO and OM, respectively.

Table 2.4 (1/7). Enumeration data from all individual sites and seasons. Bacterial counts from liquid and sediment listed as means with standard deviation of triplicates with CFU/mL or CFU/g, respectively.

				1		2		3		4		
Year	Season	Medium		liquid	sediment	liquid	sediment	liquid	sediment	liquid	sediment	
2016	Spring	RO	Total	6.55±0.75×10 ³	2.11±0.54×10 ⁵	3.40±0.40×10 ³	1.60±0.07×10 ⁴	1.01±0.22×10 ⁴	1.42±0.03×10 ⁵	5.10±1.00×10 ³	3.45±0.15×10 ³	
			Pigmented	1.90±0.40×10 ³	2.40±2.30×10 ⁴	1.40±0.20×10 ³	3.55±0.05×10 ³	1.85±0.45×10 ³	2.95±0.45×10 ⁴	3.00±0.00×10 ²	1.50±0.20×10 ³	
			AAP	2.00×10 ²	1.17×10 ⁴	3.77×10 ²	1.08×10 ³	4.32×10 ²	1.12×10 ⁴	8.68×10 ¹	1.33×10 ³	
		OM	Total	7.50±0.70×10 ⁴	1.74±0.47×10 ⁶	4.25±1.75×10 ⁴	1.31±0.42×10 ⁵	2.85±0.46×10 ⁴	4.85±0.75×10 ⁵	N/A	4.65±1.65×10 ⁵	
			Pigmented	4.50±1.50×10 ³	2.85±0.75×10 ⁵	8.00±1.00×10 ³	2.80±1.00×10 ⁴	4.30±1.30×10 ³	9.00±2.00×10 ⁴	1.90±0.40×10 ³	5.00±1.00×10 ⁴	
			AAP	7.50×10 ²	3.29×10 ⁴	4.44×10 ²	2.47×10 ⁴	3.25×10 ²	3.46×10 ³	4.75×10 ¹	1.43×10 ⁴	
		ROr	Total	2.00±1.00×10 ¹	2.31±0.36×10 ³	5.00±0.00×10 ¹	4.00±0.00×10 ¹	1.30±0.00×10 ²	4.70±0.00×10 ³	4.00±0.00×10 ¹	7.60±0.00×10 ²	
			Pigmented	1.50±0.50×10 ¹	1.87±0.17×10 ³	4.00±0.00×10 ¹	4.00±0.00×10 ¹	1.30±0.00×10 ²	1.50±0.00×10 ³	3.00±0.00×10 ¹	5.10±0.00×10 ²	
			AAP	0	6.57×10 ²	0	3.79×10 ¹	1.08×10 ¹	1.00×10 ³	9.00×10 ⁰	1.52×10 ²	
		Potato	Total	8.90±0.00×10 ²	5.45±1.45×10 ⁴	9.10±0.00×10 ²	3.50±0.00×10 ³	2.75±0.00×10 ³	4.60±0.00×10 ⁴	1.64±0.00×10 ³	8.91±0.00×10 ³	
			Pigmented	2.60±0.10×10 ²	1.35±0.75×10 ⁴	3.20±0.00×10 ²	1.00±0.00×10 ³	3.30±0.00×10 ²	6.00±0.00×10 ³	2.30±0.00×10 ²	2.80±0.00×10 ²	
			AAP	1.73×10 ¹	3.46×10 ²	3.37×10 ¹	5.00×10 ¹	6.60×10 ¹	3.53×10 ²	2.56×10 ¹	6.53×10 ¹	
		BG-11	Total	6.45±0.25×10 ⁴	7.50±1.50×10 ⁵	7.65±0.25×10 ⁴	4.16±1.98×10 ⁴	1.70±1.41×10 ⁴	3.02±0.14×10 ⁵	5.15±1.25×10 ⁴	3.25±0.15×10 ⁵	
			AOP	1.50±1.50×10 ¹	4.60±0.60×10 ²	8.50±5.50×10 ¹	1.00±0.30×10 ³	6.00±2.00×10 ²	2.05±0.05×10 ⁴	5.00±5.00×10 ¹	5.00±5.00×10 ²	
			PNSm	Total	4.35±2.25×10 ²	7.40±0.00×10 ³	2.40±0.00×10 ²	9.10±0.00×10 ²	1.70±0.00×10 ²	6.30±0.00×10 ³	9.00±0.00×10 ¹	8.20±0.00×10 ²
		AnAnP	Total	0	0	0	0	0	0	0	0	
			Pigmented	7.50±1.50×10 ³	8.50±6.50×10 ⁴	1.50±0.30×10 ³	1.80±1.00×10 ⁶	3.25±1.15×10 ³	3.95±0.35×10 ³	5.05±2.15×10 ³	1.95±0.25×10 ³	
			AAP	1.32×10 ³	1.23×10 ⁴	3.46×10 ²	8.00×10 ⁴	8.92×10 ²	7.29×10 ²	1.36×10 ³	3.78×10 ²	
		OM	Total	4.05±0.45×10 ⁵	5.05±0.15×10 ⁵	4.05±1.45×10 ⁴	6.10±0.30×10 ⁷	2.02±1.95×10 ⁴	4.80±0.40×10 ⁴	1.37±0.01×10 ⁴	8.00±1.00×10 ⁴	
			Pigmented	1.50±0.30×10 ⁵	1.25±0.65×10 ⁵	1.75±0.95×10 ⁴	2.35±0.55×10 ⁷	2.05±0.25×10 ³	1.30±0.70×10 ⁴	5.30±1.80×10 ³	2.30±0.30×10 ⁴	
			AAP	1.11×10 ⁴	1.39×10 ⁴	1.22×10 ³	0	1.58×10 ²	5.80×10 ²	4.16×10 ²	2.17×10 ³	
		ROr	Total	1.55±0.00×10 ³	3.10±0.00×10 ³	8.70±0.00×10 ²	2.41±0.00×10 ⁴	2.60±0.00×10 ²	9.60±0.00×10 ²	2.30±0.00×10 ²	5.30±0.00×10 ²	
			Pigmented	1.39±0.00×10 ³	3.10±0.00×10 ³	8.70±0.00×10 ²	2.41±0.00×10 ⁴	3.10±0.00×10 ²	8.10±0.00×10 ²	2.30±0.00×10 ²	4.90±0.00×10 ²	
			AAP	0	0	3.00×10 ¹	0	2.21×10 ¹	9.95×10 ¹	4.26×10 ¹	0	
		Potato	Total	7.90±0.00×10 ³	4.40±0.00×10 ⁴	4.00±0.00×10 ²	2.41±0.00×10 ⁴	1.05±0.00×10 ³	2.24±0.00×10 ³	1.60±0.00×10 ²	9.10±0.00×10 ³	
			Pigmented	2.70±0.00×10 ³	6.00±0.00×10 ³	1.90±0.00×10 ²	2.41±0.00×10 ⁴	3.70±0.00×10 ²	1.02±0.00×10 ³	8.00±0.00×10 ¹	1.00±0.00×10 ³	
			AAP	6.75×10 ²	7.50×10 ²	3.45×10 ¹	0	0	9.71×10 ¹	0	0	
		BG-11	Total	1.07±0.00×10 ⁶	7.50±0.00×10 ⁵	5.50±0.00×10 ⁵	4.10±0.00×10 ⁵	9.00±0.00×10 ⁵	1.49±0.00×10 ⁵	7.20±0.00×10 ³	1.58±0.00×10 ⁵	
			AOP	2.20±0.00×10 ³	2.20±0.00×10 ³	3.70±0.00×10 ²	2.00±0.00×10 ⁴	1.40±0.00×10 ⁴	4.90±0.00×10 ²	4.60±0.00×10 ²	5.00±0.00×10 ²	
			PNSm	Total	1.88±0.00×10 ³	1.27±0.00×10 ³	9.80±0.00×10 ²	4.40±0.00×10 ⁴	1.20±0.00×10 ²	1.90±0.00×10 ²	8.40±0.00×10 ²	4.50±0.00×10 ³
		AnAnP	Total	3.00±0.00×10 ¹	0	0	0	0	0	0	0	
			Pigmented	7.75±0.35×10 ³	2.44±0.14×10 ⁴	1.08±0.13×10 ⁴	5.35±0.35×10 ³	8.10±3.40×10 ³	8.95±0.25×10 ³	6.65±1.45×10 ²	1.07±0.55×10 ⁵	
			AAP	2.75±0.45×10 ³	7.50±0.50×10 ³	3.00±0.20×10 ³	1.15±0.45×10 ³	1.30±0.10×10 ³	2.10±0.60×10 ³	2.65±0.65×10 ²	2.50±0.50×10 ⁴	
	Fall	OM	Total	2.43×10 ²	4.69×10 ²	4.44×10 ²	3.38×10 ¹	1.63×10 ²	1.17×10 ²	5.68×10 ¹	0	
			Pigmented	5.95±0.65×10 ⁴	2.06±0.15×10 ⁵	4.62±0.35×10 ⁴	7.90±0.10×10 ⁴	1.80±0.09×10 ⁴	1.31±0.19×10 ⁴	2.18±0.15×10 ³	2.04±0.24×10 ⁶	
			AAP	4.50±1.50×10 ³	1.80±0.30×10 ⁴	4.00±0.10×10 ³	5.00±1.00×10 ³	1.50±0.20×10 ³	1.10±0.30×10 ³	5.00±0.30×10 ²	5.50±0.50×10 ⁵	
		ROr	Total	5.63×10 ²	0	1.74×10 ²	2.38×10 ²	9.38×10 ¹	7.33×10 ¹	4.55×10 ¹	0	
			Pigmented	3.80±0.00×10 ²	5.00±0.00×10 ³	1.18±0.00×10 ³	4.00±0.00×10 ²	1.10±0.00×10 ²	9.80±0.00×10 ²	6.00±0.00×10 ¹	1.00±0.00×10 ⁴	
			AAP	3.80±0.00×10 ²	4.50±0.00×10 ³	1.18±0.00×10 ³	3.40±0.00×10 ²	1.10±0.00×10 ²	9.00±0.00×10 ²	6.00±0.00×10 ¹	1.00±0.00×10 ⁴	
		Potato	Total	7.60×10 ¹	1.23×10 ³	1.18×10 ²	6.58×10 ¹	0	2.25×10 ²	5.00×10 ⁰	0	
			Pigmented	5.90±0.00×10 ²	2.40±0.00×10 ⁴	4.50±0.00×10 ³	1.22±0.00×10 ³	6.60±0.00×10 ²	2.40±0.00×10 ⁴	2.00±0.00×10 ¹	2.89±0.00×10 ⁶	
			AAP	2.00±0.00×10 ²	1.70±0.00×10 ⁴	1.70±0.00×10 ³	9.00±0.00×10 ²	2.20±0.00×10 ²	4.00±0.00×10 ³	0	8.60±0.00×10 ⁵	
		BG-11	Total	0	0	0	0	0	0	0	0	
			Pigmented	2.02±0.00×10 ³	1.02±0.00×10 ⁵	2.50±0.00×10 ⁵	2.17±0.00×10 ⁴	4.00±0.20×10 ⁴	9.20±0.20×10 ⁴	5.80±0.80×10 ⁴	1.35±0.28×10 ⁵	
			AOP	1.00±0.00×10 ¹	2.00±0.00×10 ²	2.70±0.00×10 ²	1.70±0.00×10 ²	7.00±5.00×10 ¹	1.80±0.10×10 ²	1.00±0.00×10 ¹	5.50±1.50×10 ¹	
		PNSm	Total	3.10±0.00×10 ²	7.00±0.00×10 ²	3.50±0.00×10 ²	1.70±0.00×10 ²	6.00±0.00×10 ¹	2.20±0.00×10 ²	2.00±0.00×10 ¹	1.74±0.00×10 ³	
			AnAnP	2.00±0.00×10 ¹	5.00±0.00×10 ¹	0	0	0	0	0	0	
	2017	Spring	RO	Total	7.63±2.10×10 ³	8.67±0.80×10 ⁴	1.71±0.27×10 ⁴	1.84±0.00×10 ⁴	2.20±0.04×10 ⁴	5.47±0.90×10 ⁵	1.53±0.05×10 ⁴	9.40±0.75×10 ⁵
				Pigmented	2.27±0.45×10 ³	4.33±1.85×10 ⁴	1.83±0.15×10 ³	2.17±0.40×10 ³	5.40±1.00×10 ³	1.09±0.04×10 ⁴	1.09±0.04×10 ⁴	3.37±0.70×10 ⁵
				AAP	1.62×10 ²	4.48×10 ³	0	9.85×10 ¹	2.08×10 ²	2.73×10 ³	2.73×10 ³	1.02×10 ⁵
			OM	Total	1.63±0.33×10 ⁴	2.20±0.27×10 ⁵	6.93±1.20×10 ⁴	1.63±0.30×10 ⁴	5.79±0.16×10 ⁴	8.77±0.10×10 ⁵	2.49±0.05×10 ⁴	1.91±0.25×10 ⁶
				Pigmented	4.40±0.85×10 ³	5.05±0.25×10 ⁴	1.10±0.65×10 ⁴	4.70±1.70×10 ³	7.83±0.90×10 ³	4.33±0.65×10 ⁵	1.19±0.13×10 ⁴	3.40±0.00×10 ⁵
				AAP	5.50×10 ²	0	5.50×10 ²	2.14×10 ²	5.11×10 ³	1.63×10 ⁵	6.78×10 ³	8.50×10 ⁴
			ROr	Total	6.67±0.00×10 ⁰	2.47±1.70×10 ²	6.23±0.95×10 ²	5.43±0.25×10 ²	4.47±2.25×10 ²	2.67±0.55×10 ²	1.40±0.15×10 ²	1.07±0.55×10 ³
				Pigmented	3.33±5.00×10 ⁰	2.37±1.55×10 ²	4.90±0.65×10 ²	4.87±0.35×10 ²	3.73±3.20×10 ²	4.27±0.30×10 ²	1.23±0.10×10 ²	8.33±3.00×10 ²
				AAP	0	1.18×10 ²	2.45×10 ²	3.35×10 ²	1.87×10 ²	1.42×10 ²	5.29×10 ¹	7.69×10 ²
			Potato	Total	6.80±0.45×10 ³	1.08±0.13×10 ⁴	2.13±0.30×10 ³	1.00±0.09×10 ⁵	5.97±0.85×10 ³	2.83±0.30×10 ³	6.10±1.40×10 ³	3.07±0.05×10 ⁴
				Pigmented	8.40±0.70×10 ³	1.38±0.09×10 ⁴	4.57±0.65×10 ³	1.47±0.04×10 ⁵	2.12±0.81×10 ⁴	3.07±0.20×10 ⁴	2.45±0.06×10 ⁴	3.37±0.10×10 ⁴
				AAP	1.20×10 ³	5.52×10 ²	4.57×10 ²	3.50×10 ⁴	1.18×10 ³	5.12×10 ³	5.52×10 ³	3.37×10 ³
			BG-11	Total	1.58±0.12×10 ⁴	1.42±0.10×10 ⁵	1.25±0.00×10 ⁵	1.80±0.00×10 ⁵	1.42±0.03×10 ⁵	1.10±0.04×10 ⁶	3.09±0.40×10 ⁴	1.75±0.21×10 ⁶
AOP		3.33±5.00×10 ⁰		3.93±0.60×10 ²	9.85±9.75×10 ³	1.45±0.05×10 ³	2.23±0.45×10 ²	1.83±0.10×10 ⁴	9.00±0.00×10 ¹	2.89±3.00×10 ³		
PNSm		Total		9.67±3.50×10 ¹	4.00±0.80×10 ²	9.70±1.20×10 ¹	1.66±1.04×10 ¹	9.37±1.10×10 ²	2.17±0.44×10 ³	1.77±0.05×10 ²	1.76±0.24×10 ⁴	
Summer		RO	Total	5.95±0.85×10 ⁴	2.80±0.80×10 ⁵	4.85±0.35×10 ³	5.15±0.55×10 ⁶	6.65±0.55×10 ³	9.60±2.20×10 ³	7.15±3.75×10 ³	1.37±0.04×10 ⁴	
			Pigmented	7.50±1.50×10 ³	8.50±6.50×10 ⁴	1.50±0.30×10 ³	1.80±1.00×10 ⁶	3.25±1.15×10 ³	3.95±0.35×10 ³	5.05±2.15×10 ³	1.95±0.25×10 ³	
			AAP	1.32×10 ³	1.23×10 ⁴	3.46×10 ²	8.00×10 ⁴	8.92×10 ²	7.29×10 ²	1.36×10 ³	3.78×10 ²	
		OM	Total	4.05±0.45×10 ⁵	5.05±0.15×10 ⁵	4.05±1.45×10 ⁴	6.10±0.30×10 ⁷	2.02±10±				

Table 2.4. Continued (2/7).

				1		2		3		4		
Year	Season	Medium		liquid	sediment	liquid	sediment	liquid	sediment	liquid	sediment	
2017	Summer	RO	Total	6.30±0.50×10 ³	8.67±0.50×10 ⁴	4.00±0.00×10 ⁴	4.27±0.55×10 ⁶	1.27±0.04×10 ³	4.03±0.15×10 ⁵	6.53±0.15×10 ³	4.75±0.00×10 ⁴	
			Pigmented	6.30±0.50×10 ³	1.80±0.35×10 ⁴	4.10±0.00×10 ⁴	7.00±1.50×10 ²	3.00±0.15×10 ²	1.20±0.15×10 ⁵	1.87±0.10×10 ³	1.35±0.00×10 ⁴	
			AAP	6.67×10 ¹	0	0	4.67×10 ⁴	3.53×10 ¹	1.55×10 ⁴	2.67×10 ²	3.21×10 ³	
		OM	Total	3.80±0.95×10 ⁵	1.14±0.09×10 ⁶	1.00±0.05×10 ⁵	5.83±2.05×10 ⁶	1.51±0.12×10 ⁴	9.83±2.30×10 ⁵	7.87±0.10×10 ⁴	1.74±0.45×10 ⁵	
			Pigmented	2.90±0.30×10 ³	1.93±0.20×10 ⁵	5.87±1.35×10 ⁴	7.00±3.00×10 ⁵	2.40±1.20×10 ³	1.13±0.45×10 ⁵	6.33±2.50×10 ³	1.43±0.45×10 ⁴	
			AAP	0	0	1.43×10 ³	5.83×10 ⁴	1.41×10 ²	3.97×10 ⁴	4.22×10 ²	8.96×10 ¹	
		RO _T	Total	9.33±0.00×10 ¹	1.24±0.21×10 ³	3.23±0.10×10 ²	2.13±3.05×10 ⁴	2.00±0.00×10 ¹	6.03±0.39×10 ³	5.20±0.25×10 ²	6.73±0.05×10 ²	
			Pigmented	9.00±0.50×10 ¹	1.24±0.21×10 ³	3.23±0.10×10 ²	2.11±0.30×10 ⁴	1.00±0.00×10 ¹	5.00±3.25×10 ³	4.30±0.20×10 ²	4.07±0.05×10 ²	
			AAP	1.50×10 ¹	3.54×10 ²	0	1.40×10 ⁴	0	1.43×10 ³	0	2.71×10 ²	
		Potato	Total	2.40±0.71×10 ³	2.34±0.24×10 ⁴	1.53±0.12×10 ⁴	1.36±0.17×10 ⁶	3.53±0.05×10 ³	3.43±0.05×10 ⁵	9.23±2.00×10 ³	4.03±0.50×10 ⁴	
			Pigmented	3.43±1.40×10 ²	3.17±0.20×10 ³	5.57±2.10×10 ³	2.03±0.60×10 ⁵	1.43±0.20×10 ³	8.67±1.00×10 ⁴	5.37±3.20×10 ³	1.57±0.10×10 ⁴	
			AAP	0	0	0	1.20×10 ⁴	6.83×10 ¹	8.39×10 ³	2.98×10 ²	0	
		BG-11	Total	5.80±1.00×10 ⁵	2.71±0.38×10 ⁵	1.14±0.31×10 ⁵	6.53±0.75×10 ⁶	2.15±0.03×10 ⁴	1.51±0.15×10 ⁶	1.01±0.02×10 ⁵	1.70±0.11×10 ⁵	
			AOP	0	0	4.60±2.10×10 ⁴	1.10±0.15×10 ⁵	3.33±5.00×10 ¹	0	1.47±0.45×10 ²	1.00±0.15×10 ³	
			PNSm	Total	2.50±0.20×10 ²	1.74±0.18×10 ³	1.36±0.10×10 ³	9.00±0.50×10 ⁴	7.00±0.00×10 ¹	1.65±0.53×10 ³	1.08±0.30×10 ³	4.13±0.20×10 ³
		Fall	RO	AnAnP	0	0	0	0	0	0	0	0
	Total			1.81±0.12×10 ⁴	5.35±0.50×10 ⁵	1.58±0.10×10 ⁴	9.91±1.53×10 ⁴	2.08±0.01×10 ⁴	3.01±0.12×10 ⁵	8.90±2.45×10 ³	1.36±0.27×10 ⁶	
	Pigmented			4.23±0.25×10 ³	1.83±0.28×10 ⁵	1.25±0.13×10 ⁴	7.44±1.17×10 ⁴	8.73±0.15×10 ³	1.14±0.00×10 ⁵	4.40±0.30×10 ³	5.97±1.10×10 ⁵	
	OM		AAP	2.65×10 ²	0	3.38×10 ²	0	3.49×10 ²	1.63×10 ⁴	2.10×10 ²	2.44×10 ⁴	
			Total	1.38±0.13×10 ⁵	6.75±0.15×10 ⁵	1.83±0.19×10 ⁴	2.80±0.00×10 ⁵	2.80±0.11×10 ⁴	1.06±0.11×10 ⁶	1.17±0.14×10 ⁴	6.13±0.15×10 ⁶	
			Pigmented	2.47±0.70×10 ⁴	1.12±0.35×10 ⁵	8.53±0.55×10 ³	7.33±0.00×10 ⁴	8.47±1.80×10 ³	4.21±0.50×10 ⁵	2.87±0.20×10 ³	1.93±0.15×10 ⁶	
	RO _T		AAP	7.47×10 ²	3.60×10 ³	0	2.53×10 ³	0	3.83×10 ⁴	1.79×10 ²	0	
			Total	8.33±1.00×10 ¹	5.80±0.33×10 ³	3.67±0.50×10 ¹	1.76±0.10×10 ³	9.00±5.00×10 ¹	8.14±0.79×10 ²	8.00±2.00×10 ¹	6.53±0.90×10 ³	
			Pigmented	8.33±1.00×10 ¹	5.48±0.45×10 ³	3.67±0.50×10 ¹	1.62±0.03×10 ³	9.00±5.00×10 ¹	7.14±0.14×10 ²	7.67±1.50×10 ¹	5.27±0.95×10 ³	
	Potato		AAP	1.19×10 ²	4.98×10 ²	9.17×10 ⁰	5.41×10 ²	1.13×10 ¹	2.60×10 ²	7.69×10 ¹	2.15×10 ³	
			Total	1.42±0.00×10 ⁴	3.17±0.23×10 ⁵	8.80±0.60×10 ³	9.27±0.10×10 ⁴	9.93±1.05×10 ³	3.08±0.00×10 ⁴	1.48±0.05×10 ³	8.93±0.80×10 ⁵	
			Pigmented	4.25±0.00×10 ³	1.33±0.20×10 ⁵	6.10±0.00×10 ³	7.67±0.13×10 ⁴	5.33±0.90×10 ³	1.49±0.00×10 ⁴	8.50±0.15×10 ²	4.00±0.75×10 ⁵	
	BG-11		AAP	1.33×10 ²	0	0	0	2.05×10 ²	5.50×10 ²	4.25×10 ¹	5.22×10 ⁴	
			Total	2.02±0.17×10 ⁴	6.75±0.15×10 ⁵	1.80±0.37×10 ⁵	1.49±0.05×10 ⁵	1.33±0.11×10 ⁵	7.76±0.86×10 ⁵	8.10±0.30×10 ⁴	1.17±0.00×10 ⁵	
			AOP	0.67±1.00×10 ²	6.67±0.25×10 ³	1.33±0.40×10 ⁴	1.00±0.17×10 ³	2.73±0.60×10 ⁴	3.81±0.71×10 ¹	5.00±0.25×10 ¹	1.50±0.65×10 ²	
	PNSm		Total	8.83±3.85×10 ²	1.07±0.21×10 ⁴	1.40±0.10×10 ²	1.84±0.10×10 ³	4.00±1.00×10 ¹	6.29±0.00×10 ²	7.33±2.50×10 ¹	6.17±0.30×10 ⁴	
		AnAnP	0	5.00±5.00×10 ¹	0	0	0	0	0	1.00±0.50×10 ¹		
2018	Spring	RO	Total	6.47±3.85×10 ³	1.37±0.20×10 ⁵	4.53±0.30×10 ⁴	1.13±0.18×10 ⁵	1.38±0.02×10 ³	6.05±0.00×10 ⁴	3.33±1.75×10 ²	2.97±1.10×10 ⁵	
			Pigmented	2.67±1.20×10 ⁴	5.00±0.50×10 ⁴	1.30±0.45×10 ⁵	4.87±1.60×10 ⁴	3.87±0.30×10 ³	2.07±1.60×10 ⁴	1.50±1.10×10 ³	6.23±0.15×10 ⁴	
			AAP	4.64×10 ³	4.63×10 ³	2.60×10 ⁴	2.05×10 ⁴	1.10×10 ³	2.02×10 ³	8.33×10 ¹	1.47×10 ⁴	
		OM	Total	1.11±0.56×10 ⁴	2.00±0.90×10 ⁵	6.87±1.40×10 ⁴	1.60±0.01×10 ⁵	3.00±0.00×10 ⁴	1.35±0.08×10 ⁵	2.59±0.72×10 ³	3.70±0.20×10 ⁵	
			Pigmented	3.83±2.65×10 ⁴	4.67±1.50×10 ⁴	7.00±1.00×10 ⁴	4.93±0.25×10 ⁴	3.00±0.00×10 ⁴	1.83±0.25×10 ⁴	4.00±0.70×10 ³	5.03±0.20×10 ⁴	
			AAP	0	1.67×10 ³	5.71×10 ³	0	2.73×10 ³	3.67×10 ³	4.44×10 ²	8.39×10 ³	
		RO _T	Total	8.00±2.00×10 ¹	4.43±0.35×10 ²	6.63±3.80×10 ²	1.46±0.95×10 ³	5.33±3.50×10 ¹	1.20±0.45×10 ²	3.00±1.50×10 ¹	3.00±0.58×10 ³	
			Pigmented	8.00±2.00×10 ¹	2.23±0.00×10 ²	5.77±3.60×10 ²	1.22±0.75×10 ³	2.67±0.50×10 ¹	9.67±2.50×10 ¹	3.00±1.50×10 ¹	3.00±0.58×10 ³	
			AAP	0	1.49×10 ²	2.88×10 ²	8.59×10 ²	7.06×10 ⁰	9.67×10 ¹	0	2.18×10 ³	
		Potato	Total	5.53±0.75×10 ²	8.33±1.30×10 ²	5.17±0.25×10 ²	7.30±7.60×10 ³	1.74±0.22×10 ³	1.10±0.00×10 ⁴	2.25±1.04×10 ³	3.60±1.15×10 ³	
			Pigmented	4.33±1.10×10 ²	4.60±0.45×10 ²	2.37±0.50×10 ²	4.43±4.65×10 ³	5.37±1.10×10 ²	2.50±0.00×10 ³	4.90±2.95×10 ²	1.17±0.04×10 ³	
			AAP	8.46×10 ¹	6.97×10 ¹	1.23×10 ²	2.55×10 ³	3.22×10 ²	1.07×10 ³	8.91×10 ¹	3.55×10 ²	
		BG-11	Total	4.33±0.60×10 ⁴	8.70±0.10×10 ⁴	9.83±4.00×10 ⁵	1.29±1.12×10 ⁵	3.43±0.30×10 ⁵	5.15±0.00×10 ⁵	7.75±0.00×10 ⁴	1.95±0.06×10 ⁵	
			AOP	1.13±0.35×10 ²	3.97±0.45×10 ²	1.63±0.05×10 ³	1.83±0.25×10 ³	1.40±0.25×10 ²	2.05±0.00×10 ³	1.90±1.50×10 ²	1.80±0.30×10 ³	
			PNSm	Total	1.13±0.20×10 ²	8.33±4.50×10 ¹	2.43±0.40×10 ²	1.40±0.16×10 ³	1.90±0.55×10 ²	1.33±0.02×10 ³	1.33±0.00×10 ¹	1.90±0.20×10 ²
		Summer	RO	AnAnP	0	0	0	0	0	0	0	0
	Total			1.15±0.07×10 ⁴	1.36±0.24×10 ⁴	1.13±0.19×10 ⁴	5.53±1.00×10 ⁴	2.63±0.35×10 ⁴	9.33±1.25×10 ⁵	1.36±0.11×10 ⁴	3.93±0.10×10 ⁴	
	Pigmented			8.33±3.50×10 ²	3.75±1.35×10 ³	2.33±0.35×10 ³	2.17±0.55×10 ⁴	1.17±0.25×10 ⁴	4.10±0.65×10 ⁵	4.93±0.35×10 ³	1.77±0.05×10 ⁴	
	OM		AAP	0	0	1.40×10 ³	3.61×10 ³	1.89×10 ³	8.33×10 ⁴	1.50×10 ³	0	
			Total	1.45±0.00×10 ⁴	9.10±1.30×10 ⁴	3.34±0.15×10 ⁴	1.10±0.05×10 ⁵	4.73±0.75×10 ⁴	2.45±0.06×10 ⁶	2.36±0.12×10 ⁴	1.10±0.02×10 ⁵	
			Pigmented	1.30±0.00×10 ³	3.10±0.30×10 ⁴	6.43±0.20×10 ³	3.43±0.55×10 ⁴	1.33±0.35×10 ⁴	9.77±0.60×10 ⁵	7.27±0.20×10 ³	2.63±0.65×10 ⁴	
	RO _T		AAP	0	5.64×10 ³	1.77×10 ³	0	3.46×10 ³	1.87×10 ⁵	3.85×10 ³	0	
			Total	9.67±8.50×10 ¹	3.53±0.85×10 ²	3.03±1.40×10 ²	9.67±6.50×10 ¹	1.50±0.50×10 ²	2.07±1.00×10 ³	4.17±0.45×10 ²	9.50±6.10×10 ²	
			Pigmented	9.67±8.50×10 ¹	2.87±0.65×10 ²	2.13±1.90×10 ²	5.00±2.50×10 ¹	1.50±0.50×10 ²	2.00±0.90×10 ³	3.83±0.25×10 ²	7.63±5.20×10 ²	
	Potato		AAP	0	0	1.90×10 ²	0	2.31×10 ¹	5.38×10 ²	1.92×10 ²	0	
			Total	7.33±0.70×10 ³	1.28±0.10×10 ³	8.30±1.75×10 ³	1.73±0.08×10 ⁴	8.17±1.45×10 ³	4.50±1.15×10 ⁵	6.10±1.30×10 ³	8.67±1.40×10 ³	
			Pigmented	7.00±2.00×10 ²	3.20±0.50×10 ²	2.83±0.85×10 ³	7.07±0.25×10 ³	3.77±0.25×10 ³	3.43±0.35×10 ⁵	3.50±0.90×10 ³	4.47±1.30×10 ³	
	BG-11		AAP	0	0	5.15×10 ²	0	9.83×10 ²	4.64×10 ⁴	7.50×10 ²	0	
			Total	1.23±0.07×10 ⁴	1.89±0.25×10 ⁴	1.33±0.50×10 ⁴	1.00±0.00×10 ⁴	1.17±0.40×10 ⁵	1.00±0.50×10 ⁷	8.67±1.00×10 ³	1.00±1.00×10 ⁷	
			AOP	0	0	1.57±0.20×10 ²	1.27±0.05×10 ³	5.07±0.85×10 ²	1.43±0.20×10 ⁴	2.35±0.00×10 ²	1.27±1.50×10 ³	
	PNSm		Total	7.10±0.20×10 ³	1.57±0.09×10 ³	2.54±0.27×10 ³	1.22±1.70×10 ³	2.40±0.16×10 ³	3.93±0.60×10 ⁴	9.10±0.15×10 ²	1.06±1.20×10 ³	
		AnAnP	0	0	1.80±0.50×10 ²	0	0	0	0	0		

Table 2.4. Continued (3/7).

				1		2		3		4	
Year	Season	Medium		liquid	sediment	liquid	sediment	liquid	sediment	liquid	sediment
2018	Fall	RO	Total	1.86±0.98×10 ⁴	1.92±0.04×10 ⁴	6.27±0.15×10 ⁴	3.60±0.55×10 ⁴	1.25±0.01×10 ⁴	5.70±0.20×10 ³	1.89±0.23×10 ⁴	1.38±0.26×10 ⁴
			Pigmented	1.63±0.50×10 ³	3.60±0.05×10 ³	1.43±0.00×10 ³	1.70±0.50×10 ⁴	4.70±0.15×10 ³	2.60±0.20×10 ³	7.63±0.05×10 ³	6.27±0.95×10 ³
			AAP	6.53×10 ¹	3.72×10 ²	2.61×10 ³	4.47×10 ²	2.61×10 ²	2.74×10 ²	3.55×10 ²	4.18×10 ²
		OM	Total	3.87±0.15×10 ⁴	7.47±0.10×10 ⁴	1.17±0.04×10 ⁴	7.90±0.10×10 ⁴	4.57±0.65×10 ⁴	1.41±0.01×10 ⁴	3.00±0.13×10 ⁴	3.87±0.15×10 ⁴
			Pigmented	3.67±0.00×10 ³	1.13±0.50×10 ⁴	4.83±0.60×10 ³	2.17±0.10×10 ⁴	1.47±0.35×10 ⁴	3.30±0.25×10 ³	8.37±0.35×10 ³	1.10±0.10×10 ⁴
			AAP	0	0	0	1.11×10 ³	1.83×10 ³	0	1.01×10 ³	7.86×10 ²
		RO _r	Total	8.67±0.50×10 ¹	5.70±0.65×10 ²	5.80±0.90×10 ²	1.66±0.12×10 ³	4.47±0.55×10 ²	1.13±0.05×10 ²	9.67±1.50×10 ¹	7.77±0.70×10 ²
			Pigmented	8.67±0.50×10 ¹	5.60±0.60×10 ²	5.80±0.90×10 ²	1.66±0.12×10 ³	4.47±0.55×10 ²	1.13±0.05×10 ²	8.67±0.00×10 ¹	7.77±0.70×10 ²
			AAP	1.73×10 ¹	0	2.42×10 ²	5.19×10 ²	5.58×10 ¹	0	6.67×10 ⁰	2.59×10 ²
		Potato	Total	4.77±0.15×10 ³	9.70±0.25×10 ²	1.12±0.06×10 ³	4.63±0.05×10 ³	9.37±2.05×10 ³	2.73±0.35×10 ³	9.63±0.70×10 ³	9.70±0.40×10 ³
			Pigmented	2.43±0.40×10 ³	2.10±0.25×10 ²	4.57±0.50×10 ²	2.13±0.30×10 ³	4.23±0.60×10 ³	1.33±0.50×10 ³	5.93±0.70×10 ³	6.30±0.05×10 ³
			AAP	1.43×10 ²	0	8.30×10 ¹	1.94×10 ²	0	0	0	0
		BG-11	Total	2.39±0.18×10 ⁴	5.57±1.95×10 ⁴	1.70±0.11×10 ⁴	3.30±0.61×10 ⁴	2.10±0.50×10 ⁴	4.03±0.45×10 ⁵	6.60±2.70×10 ³	2.26±0.35×10 ⁶
			AOP	3.30±0.00×10 ⁰	2.67±2.60×10 ²	8.33±2.00×10 ¹	2.13±0.45×10 ²	2.35±0.00×10 ²	6.57±4.70×10 ³	1.23±0.35×10 ²	2.57±0.85×10 ⁴
			PNSm	Total	8.00±0.00×10 ¹	1.27±0.20×10 ²	1.10±0.05×10 ²	9.00±0.50×10 ¹	1.53±0.05×10 ²	2.40±0.30×10 ²	7.33±0.50×10 ¹
			AnAnP	6.67±0.00×10 ⁰	3.33±1.00×10 ¹	0	0	0	0	0	0
2019	Spring	RO	Total	2.01±0.52×10 ³	1.23±0.26×10 ⁴	3.13±0.20×10 ³	5.05±0.00×10 ⁴	1.13±0.03×10 ⁴	8.53±0.15×10 ³	1.36±0.15×10 ⁴	4.40±0.45×10 ⁵
			Pigmented	3.80±1.20×10 ²	5.07±3.45×10 ³	1.33±0.15×10 ³	6.75±0.00×10 ³	4.13±0.10×10 ³	4.30±0.95×10 ³	4.37±0.05×10 ³	2.83±0.00×10 ⁵
			AAP	1.22×10 ²	1.97×10 ³	3.01×10 ²	3.21×10 ³	1.22×10 ³	2.04×10 ³	1.28×10 ³	1.77×10 ⁵
		OM	Total	1.87±0.43×10 ⁴	4.53±0.90×10 ⁴	4.03±0.45×10 ⁴	4.77±0.95×10 ⁵	8.90±2.65×10 ³	3.41±0.16×10 ⁵	4.97±0.00×10 ⁴	7.27±1.15×10 ⁵
			Pigmented	2.93±0.80×10 ³	5.33±0.50×10 ³	4.67±1.00×10 ³	6.67±1.50×10 ⁴	3.03±0.60×10 ³	5.03±0.15×10 ⁴	5.67±0.50×10 ³	1.90±0.20×10 ⁵
			AAP	6.67×10 ²	1.11×10 ³	2.03×10 ³	2.42×10 ⁴	5.59×10 ²	1.53×10 ⁴	1.47×10 ³	9.07×10 ⁴
		RO _r	Total	3.97±1.85×10 ²	5.00±1.80×10 ²	6.67±0.70×10 ²	3.05±0.03×10 ³	2.25±0.00×10 ²	3.23±0.67×10 ³	1.43±0.07×10 ²	2.03±0.26×10 ³
			Pigmented	3.63±1.55×10 ²	5.00±1.80×10 ²	5.23±0.45×10 ²	2.94±0.19×10 ³	1.15±0.00×10 ²	1.91±0.88×10 ³	1.27±0.45×10 ²	1.02±0.77×10 ³
			AAP	3.63×10 ²	3.00×10 ²	3.49×10 ²	1.07×10 ³	2.30×10 ¹	4.78×10 ²	0	6.14×10 ²
		Potato	Total	3.52±0.07×10 ³	2.22±0.01×10 ³	9.10±0.00×10 ³	7.43±0.85×10 ⁴	8.27±1.60×10 ³	2.37±0.01×10 ⁴	3.53±0.50×10 ³	4.50±0.45×10 ⁴
			Pigmented	5.67±1.10×10 ²	4.50±0.05×10 ²	3.35±0.00×10 ³	2.70±0.35×10 ⁴	3.80±1.05×10 ³	1.30±0.02×10 ⁴	2.53±0.15×10 ³	2.77±0.00×10 ⁴
			AAP	2.48×10 ²	1.31×10 ²	1.73×10 ³	1.35×10 ⁴	2.03×10 ³	1.13×10 ⁴	1.86×10 ³	2.40×10 ⁴
		R2A	Total	N/A	3.25±0.61×10 ⁴	5.53±0.60×10 ⁴	6.30±3.00×10 ⁴	5.90±0.05×10 ⁴	5.13±0.05×10 ⁵	2.09±0.22×10 ⁴	2.22±0.19×10 ⁴
			Pigmented	N/A	7.47±1.65×10 ³	9.67±0.45×10 ³	2.80±1.80×10 ⁴	1.70±0.35×10 ⁴	2.00±0.45×10 ⁵	7.60±1.05×10 ³	8.17±1.15×10 ⁴
			AAP	N/A	1.74×10 ³	2.94×10 ³	1.01×10 ⁴	1.79×10 ³	1.36×10 ⁵	5.43×10 ³	4.67×10 ⁴
		BG-11	Total	2.14±0.74×10 ⁴	4.45±1.25×10 ⁴	1.07±0.02×10 ⁵	2.30±0.06×10 ⁵	4.57±0.00×10 ⁴	1.00±0.10×10 ⁶	5.33±0.65×10 ⁴	4.57±0.20×10 ⁵
			AOP	1.30±0.05×10 ²	5.33±0.50×10 ¹	0	3.50±0.30×10 ⁴	0	4.43±2.05×10 ⁴	2.33±0.00×10 ¹	4.87±0.40×10 ²
			PNSm	Total	5.77±2.35×10 ²	2.67±0.00×10 ¹	3.83±0.10×10 ²	1.32±0.04×10 ⁴	1.05±0.05×10 ³	3.43±0.10×10 ³	5.67±1.50×10 ¹
			AnAnP	4.33±2.50×10 ¹	0	0	0	1.33±2.00×10 ¹	3.33±5.00×10 ¹	0	1.00±1.50×10 ¹
	Summer	RO	Total	5.63±1.50×10 ³	5.77±0.75×10 ⁴	7.50±3.10×10 ⁴	4.40±0.75×10 ⁶	6.80±0.30×10 ⁴	1.05±0.07×10 ⁵	9.63±0.50×10 ³	2.48±0.26×10 ⁵
			Pigmented	3.07±0.60×10 ³	0	2.40±0.25×10 ⁴	2.03±0.30×10 ⁵	2.93±0.25×10 ⁴	5.07±0.30×10 ⁴	3.33±0.40×10 ³	8.20±0.95×10 ⁴
			AAP	6.13×10 ²	0	5.19×10 ³	4.15×10 ⁴	6.29×10 ³	1.27×10 ⁴	4.76×10 ²	2.39×10 ⁴
		OM	Total	4.57±0.20×10 ⁴	N/A	1.43±0.16×10 ⁵	6.40±2.90×10 ⁶	5.73±1.65×10 ⁴	3.37±0.15×10 ⁵	3.36±0.11×10 ⁴	1.11±0.13×10 ⁶
			Pigmented	5.00±1.00×10 ³	N/A	2.73±1.20×10 ⁴	1.97±0.45×10 ⁵	2.03±0.30×10 ⁴	1.50±0.45×10 ⁵	2.47±0.35×10 ³	1.87±0.20×10 ⁵
			AAP	0	0	3.28×10 ³	4.57×10 ⁴	0	1.10×10 ⁴	5.23×10 ²	1.78×10 ⁴
		RO _r	Total	7.17±2.80×10 ²	4.17±1.25×10 ³	1.70±0.03×10 ³	5.07±1.20×10 ⁴	1.22±0.20×10 ³	3.92±0.29×10 ³	1.77±0.50×10 ²	9.83±0.45×10 ³
			Pigmented	7.13±2.75×10 ²	2.87±1.20×10 ³	1.51±0.01×10 ³	2.57±0.10×10 ³	7.20±2.20×10 ²	3.42±0.32×10 ³	1.77±0.50×10 ²	5.93±0.95×10 ³
			AAP	1.43×10 ²	0	1.08×10 ³	1.14×10 ⁴	0	2.63×10 ³	1.33×10 ²	4.94×10 ³
		Potato	Total	1.43±0.15×10 ⁴	9.47±0.85×10 ³	5.60±0.80×10 ⁴	1.05±0.44×10 ⁵	6.00±3.80×10 ³	4.57±0.35×10 ⁴	1.54±0.13×10 ³	3.44±0.99×10 ⁴
			Pigmented	2.67±0.40×10 ³	3.37±0.40×10 ³	2.27±0.10×10 ⁴	4.23±1.15×10 ⁵	2.83±0.65×10 ³	2.70±0.35×10 ⁴	4.93±0.60×10 ²	9.37±3.55×10 ³
			AAP	2.67×10 ²	0	2.06×10 ³	7.70×10 ⁴	5.83×10 ²	4.70×10 ³	1.37×10 ²	2.04×10 ³
		R2A	Total	1.56±0.03×10 ⁴	4.70±0.40×10 ⁴	1.31±0.25×10 ⁵	9.93±0.30×10 ⁵	1.23±0.05×10 ⁵	1.00±0.18×10 ⁵	3.35±0.42×10 ⁴	8.30±1.30×10 ⁵
			Pigmented	1.80±0.25×10 ³	6.00±0.50×10 ²	3.50±0.30×10 ⁴	4.53±0.55×10 ⁵	4.77±1.55×10 ⁵	5.30±1.70×10 ⁴	5.30±1.02×10 ³	1.02±0.13×10 ⁴
			AAP	0	2.86×10 ²	0	9.71×10 ⁴	2.38×10 ³	4.82×10 ³	1.20×10 ³	2.50×10 ⁴
		BG-11	Total	1.43±0.07×10 ⁴	3.92±0.26×10 ⁴	5.17±1.00×10 ⁵	6.87±0.40×10 ⁶	1.21±0.07×10 ⁵	5.23±0.10×10 ⁵	8.60±2.85×10 ⁴	4.97±0.95×10 ⁶
			AOP	4.44±1.33×10 ³	1.37±0.03×10 ³	8.57±0.00×10 ³	1.33±0.05×10 ⁴	7.20±0.10×10 ²	2.33±1.20×10 ²	8.47±6.00×10 ²	1.70±0.14×10 ⁴
			PNSm	Total	1.33±0.50×10 ¹	2.67±0.50×10 ¹	6.83±0.55×10 ²	4.23±0.65×10 ²	3.33±0.75×10 ¹	1.99±0.07×10 ³	9.47±0.10×10 ³
			AnAnP	0	0	1.50±0.10×10 ²	0	0	0	1.67±0.00×10 ¹	0
	Fall	RO	Total	1.13±0.08×10 ⁴	5.07±0.40×10 ⁴	4.47±0.05×10 ³	8.17±0.15×10 ³	1.76±0.84×10 ⁴	6.80±0.55×10 ⁴	9.43±1.95×10 ³	5.47±0.15×10 ⁵
			Pigmented	2.97±0.60×10 ³	2.03±0.30×10 ⁴	2.77±0.55×10 ³	2.70±0.05×10 ³	3.87±1.70×10 ³	3.20±0.60×10 ⁴	3.03±1.60×10 ³	2.80±0.00×10 ⁵
			AAP	2.97×10 ²	1.36×10 ³	0	5.79×10 ²	8.79×10 ²	1.01×10 ⁴	4.33×10 ²	6.67×10 ³
		OM	Total	1.71±0.04×10 ⁴	2.46±0.19×10 ⁵	6.03±0.15×10 ⁴	6.83±2.10×10 ⁴	1.57±0.19×10 ⁴	1.36±0.09×10 ⁵	4.43±0.25×10 ⁴	4.70±0.15×10 ⁶
			Pigmented	4.25±1.85×10 ³	2.63±0.35×10 ⁴	5.33±0.50×10 ³	1.53±0.30×10 ⁴	2.13±0.35×10 ³	2.90±0.60×10 ⁴	9.33±2.50×10 ³	147±0.05×10 ⁶
			AAP	7.08×10 ²	6.58×10 ³	6.27×10 ²	2.19×10 ³	5.33×10 ²	2.90×10 ³	1.65×10 ³	4.73×10 ⁴
RO _r		Total	1.08±0.02×10 ³	8.30±1.45×10 ³	1.56±0.10×10 ³	6.50±0.65×10 ²	6.60±0.25×10 ²	9.63±2.00×10 ²	7.25±0.00×10 ²	2.60±0.40×10 ⁴	
		Pigmented	8.03±2.45×10 ²	8.10±1.30×10 ³	1.24±0.10×10 ³	4.83±0.15×10 ²	3.30±0.20×10 ²	7.97±1.85×10 ²	5.95±0.00×10 ²	2.60±0.40×10 ⁴	
		AAP	1.15×10 ²	0	4.97×10 ²	8.53×10 ¹	1.10×10 ²	5.98×10 ²	8.93×10 ¹	1.53×10 ⁴	
Potato		Total	1.13±0.00×10 ⁴	2.82±0.00×10 ⁴	8.80±0.00×10 ³	3.33±0.05×10 ³	4.80±3.40×10 ³	6.20±0.20×10 ⁴	7.70±0.		

Table 2.4. Continued (4/7).

Year	Season	Medium		5		6		7		8		9		10	
				liquid	sediment	liquid	liquid	liquid	liquid	liquid	liquid	liquid	liquid	liquid	liquid
2016	Spring	RO	Total	1.53±0.10×10 ³	7.90±0.00×10 ⁴	1.01±0.60×10 ⁴	1.02±0.17×10 ⁴	2.18±0.81×10 ⁴	7.50±1.20×10 ³	4.45±0.35×10 ³					
			Pigmented	3.00±0.20×10 ²	1.40±1.40×10 ⁴	4.95±1.35×10 ³	4.20±2.00×10 ³	1.24±0.01×10 ⁴	6.65±1.55×10 ³	3.70±0.00×10 ³					
			AAP	8.68×10 ¹	8.33×10 ³	7.40×10 ²	2.55×10 ²	3.92×10 ²	1.05×10 ³	2.87×10 ²					
		OM	Total	1.86±1.07×10 ⁴	2.77±0.39×10 ⁵	2.38±0.31×10 ⁴	3.35±0.35×10 ⁴	8.90±0.20×10 ³	1.38±0.14×10 ⁴	3.909±0.70×10 ³					
			Pigmented	5.35±0.35×10 ²	1.95±1.35×10 ⁴	5.50±0.30×10 ³	1.45±0.05×10 ⁴	3.40±1.60×10 ³	6.25±0.85×10 ³	1.50±0.00×10 ³					
			AAP	1.34×10 ¹	6.91×10 ³	1.59×10 ²	3.15×10 ²	4.36×10 ¹	8.12×10 ¹	2.03×10 ¹					
		RO _r	Total	1.70±0.00×10 ²	6.50±0.00×10 ²	1.10±0.10×10 ²	0	1.00±0.00×10 ¹	3.80±1.20×10 ²	2.00±0.00×10 ¹					
			Pigmented	1.50±0.00×10 ²	5.90±0.00×10 ²	1.10±0.10×10 ²	0	1.00±0.00×10 ¹	3.80±1.20×10 ²	2.00±0.00×10 ¹					
			AAP	0	4.29×10 ²	3.47×10 ¹	0	0	7.60×10 ¹	0					
		Potato	Total	7.80±0.00×10 ²	3.70±0.00×10 ⁴	1.20±0.20×10 ³	2.95±0.65×10 ³	2.50±0.60×10 ²	1.80±0.20×10 ²	3.65±0.55×10 ²					
			Pigmented	2.00±0.00×10 ²	5.00±0.00×10 ³	7.75±5.75×10 ²	1.00±1.00×10 ²	8.00±3.00×10 ¹	2.50±0.50×10 ¹	1.00±0.10×10 ²					
			AAP	2.50×10 ¹	9.09×10 ²	4.13×10 ²	1.43×10 ¹	4.71×10 ⁰	8.33×10 ⁰	0					
		BG-11	Total	2.14±0.00×10 ⁵	1.00±0.00×10 ⁶	2.31±0.84×10 ⁴	1.86±0.07×10 ⁴	1.42±1.07×10 ⁴	7.65±1.65×10 ³	N/A					
			AOP	2.40±0.00×10 ³	1.00±0.00×10 ²	1.50±0.50×10 ¹	5.00±5.00×10 ⁰	0	6.50±2.50×10 ¹	N/A					
			AAP	2.40×10 ²	1.78×10 ²	3.33×10 ¹	3.61×10 ¹	1.75×10 ¹	8.87×10 ¹	9.35×10 ¹					
		PNSm	Total	1.20±0.00×10 ²	6.90±0.00×10 ²	1.05±0.25×10 ²	1.50±0.10×10 ²	6.00±3.00×10 ¹	2.50±0.50×10 ¹	0					
			AnAnP	0	1.00±0.00×10 ¹	0	0	0	0	0					
			AAP	0	0	0	0	0	0	0					
	Summer	RO	Total	6.75±1.25×10 ³	2.01±0.24×10 ³	5.40±0.20×10 ²	3.35±0.15×10 ³	1.34±0.15×10 ³	3.05±0.05×10 ³	1.53±0.20×10 ³					
			Pigmented	2.10±0.20×10 ³	4.20±0.40×10 ²	8.00±5.00×10 ¹	2.90±0.20×10 ³	3.70±0.30×10 ²	2.60±0.30×10 ³	1.32±0.22×10 ³					
			AAP	4.20×10 ²	9.16×10 ¹	4.21×10 ⁰	3.45×10 ²	3.36×10 ¹	3.59×10 ²	8.22×10 ¹					
		OM	Total	1.89±0.27×10 ⁴	1.28±0.24×10 ⁴	1.85±0.25×10 ³	1.88±0.04×10 ³	2.73±0.04×10 ³	4.90±0.20×10 ³	4.90±1.10×10 ³					
			Pigmented	2.35±0.35×10 ³	1.35±0.85×10 ³	4.00±1.00×10 ²	1.19±0.04×10 ³	6.30±0.70×10 ²	2.75±0.55×10 ³	1.45±0.55×10 ³					
			AAP	2.40×10 ²	1.78×10 ²	3.33×10 ¹	3.61×10 ¹	1.75×10 ¹	8.87×10 ¹	9.35×10 ¹					
		RO _r	Total	7.40±0.00×10 ²	3.00±0.00×10 ²	1.10±4.00×10 ²	4.05±0.65×10 ²	3.20±0.00×10 ²	9.00±0.00×10 ¹	3.40±0.00×10 ²					
			Pigmented	7.00±0.00×10 ²	2.90±0.00×10 ²	1.10±0.00×10 ²	4.05±0.65×10 ²	1.00±0.00×10 ²	9.00±0.00×10 ¹	3.40±0.00×10 ²					
			AAP	0	0	0	9.42×10 ⁰	0	0	0					
		Potato	Total	8.88±0.00×10 ²	4.50±0.00×10 ²	2.20±0.00×10 ³	4.00±0.00×10 ³	8.00±0.00×10 ²	2.00±0.00×10 ³	5.70±0.00×10 ²					
			Pigmented	3.20±0.00×10 ²	6.00±0.00×10 ¹	1.80±0.00×10 ³	3.70±0.00×10 ³	1.90±0.00×10 ²	7.70±0.00×10 ³	1.40±0.00×10 ²					
			AAP	0	0	0	0	0	0	0					
		BG-11	Total	2.10±0.00×10 ⁵	9.00±0.00×10 ⁵	7.00±0.00×10 ³	6.70±0.00×10 ³	4.00±0.00×10 ³	4.70±0.00×10 ³	3.40±0.00×10 ³					
			AOP	4.40±0.00×10 ²	0	1.20±0.00×10 ²	1.00±0.00×10 ²	0	0	1.00±0.00×10 ²					
			AAP	4.40×10 ²	0	1.20×10 ²	1.00×10 ²	0	0	1.00×10 ²					
		PNSm	Total	4.80±0.00×10 ²	1.60±0.00×10 ²	5.00±5.00×10 ⁰	3.00±2.00×10 ¹	2.40±0.00×10 ²	1.90±0.00×10 ²	9.00±0.00×10 ¹					
			AnAnP	0	1.00±0.00×10 ¹	0	0	0	0	0					
			AAP	0	0	0	0	0	0	0					
	Fall	RO	Total	1.41±0.60×10 ⁴	1.07±0.11×10 ⁵	6.15±0.65×10 ²	8.35±1.35×10 ³	1.20±0.40×10 ²	7.45±1.65×10 ²	4.35±0.85×10 ²					
			Pigmented	4.60±4.10×10 ³	1.10±0.40×10 ⁵	4.65±0.85×10 ²	7.40±1.50×10 ³	7.50±1.50×10 ¹	3.15±0.85×10 ²	2.60±0.50×10 ²					
			AAP	8.00×10 ²	7.33×10 ³	0	3.08×10 ²	0	0	0					
		OM	Total	8.25±1.55×10 ⁴	1.15±0.45×10 ⁶	2.57±0.69×10 ³	9.80±1.60×10 ³	1.61±0.02×10 ³	5.70±0.00×10 ³	1.90±0.62×10 ³					
			Pigmented	1.35±0.15×10 ⁴	1.50±0.50×10 ⁵	4.40±0.60×10 ²	8.05±1.15×10 ³	4.10±1.30×10 ²	9.50±2.50×10 ²	3.70±1.30×10 ²					
			AAP	1.35×10 ³	0	0	0	0	0	0					
		RO _r	Total	3.80±0.00×10 ²	4.00±0.00×10 ⁴	0	3.10±0.00×10 ³	1.10±0.00×10 ²	5.00±0.00×10 ¹	1.60±0.00×10 ²					
			Pigmented	3.80±0.00×10 ²	3.90±0.00×10 ⁴	0	3.10±0.00×10 ³	1.10±0.00×10 ²	4.00±0.00×10 ¹	1.50±0.00×10 ²					
			AAP	0	0	0	0	0	0	0					
		Potato	Total	5.60±0.00×10 ³	6.50±0.00×10 ⁴	3.80±0.00×10 ²	4.30±0.00×10 ³	2.90±0.00×10 ²	2.90±0.00×10 ²	4.40±0.00×10 ²					
			Pigmented	2.10±0.00×10 ³	5.50±0.00×10 ⁴	2.00±0.00×10 ²	4.30±0.00×10 ³	2.00±0.00×10 ²	2.20±0.00×10 ²	3.90±0.00×10 ²					
			AAP	0	0	0	0	0	0	0					
		BG-11	Total	3.50±0.20×10 ⁴	3.25±0.86×10 ⁵	8.70±0.00×10 ³	1.05±0.00×10 ⁴	2.40±0.00×10 ²	1.60±0.00×10 ²	1.63±0.00×10 ⁴					
			AOP	8.50±0.50×10 ¹	5.25±0.25×10 ²	4.00±0.00×10 ¹	2.00±0.00×10 ¹	0	1.00±0.00×10 ¹	2.00±0.00×10 ¹					
			AAP	2.40×10 ²	1.78×10 ²	3.33×10 ¹	3.61×10 ¹	1.75×10 ¹	8.87×10 ¹	9.35×10 ¹					
		PNSm	Total	1.30±0.00×10 ²	1.01±0.00×10 ³	6.00±0.00×10 ¹	2.00±0.00×10 ¹	5.00±0.00×10 ¹	9.00±0.00×10 ¹	1.00±0.00×10 ¹					
			AnAnP	0	N/A	0	0	0	0	0					
			AAP	0	0	0	0	0	0	0					
2017	Winter	Alternative Sites				W10	W1	W9	W6	W8					
		RO	Total	N/A	N/A	N/A	N/A	2.00±0.00×10 ¹	N/A	N/A					
			Pigmented	N/A	N/A	N/A	N/A	1.00±0.50×10 ¹	N/A	N/A					
			AAP	N/A	N/A	N/A	N/A	0	N/A	N/A					
		OM	Total	N/A	N/A	N/A	N/A	1.44±0.50×10 ¹	N/A	N/A					
			Pigmented	N/A	N/A	N/A	N/A	3.33±0.00×10 ⁰	N/A	N/A					
			AAP	N/A	N/A	N/A	N/A	0	N/A	N/A					
		RO _r	Total	N/A	N/A	N/A	N/A	6.67±0.50×10 ⁰	N/A	N/A					
			Pigmented	N/A	N/A	N/A	N/A	3.33±0.00×10 ⁰	N/A	N/A					
			AAP	N/A	N/A	N/A	N/A	0	N/A	N/A					
		Potato	Total	N/A	N/A	N/A	N/A	3.33±0.00×10 ⁰	N/A	N/A					
			Pigmented	N/A	N/A	N/A	N/A	0	N/A	N/A					
			AAP	N/A	N/A	N/A	N/A	0	N/A	N/A					
		BG-11	Total	N/A	N/A	N/A	N/A	7.67±3.00×10 ¹	N/A	N/A					
			AOP	N/A	N/A	N/A	N/A	0	N/A	N/A					
			AAP	N/A	N/A	N/A	N/A	0	N/A	N/A					
		PNSm	Total	N/A	N/A	N/A	N/A	1.33±0.50×10 ¹	N/A	N/A					
			AnAnP	N/A	N/A	N/A	N/A	0	N/A	N/A					

Table 2.4. Continued (5/7).

				5	6	7	8	9	10					
Year	Season	Medium		liquid	sediment	liquid	liquid	liquid	liquid	liquid				
2017	Spring	RO	Total	5.10±0.25×10 ³	1.23±0.07×10 ⁴	2.09±0.97×10 ³	1.08±0.26×10 ³	1.94±0.23×10 ⁴	8.30±0.70×10 ³	6.40±0.60×10 ³				
			Pigmented	2.50±0.40×10 ³	3.80±0.25×10 ³	4.33±5.01×10 ²	9.67±1.00×10 ¹	1.44±0.48×10 ⁴	4.50±0.35×10 ³	5.20±0.20×10 ³				
			AAP	1.12×10 ³	3.68×10 ²	0	8.79×10 ⁰	1.80×10 ³	4.29×10 ²	8.67×10 ²				
		OM	Total	1.23±0.03×10 ⁴	7.97±0.40×10 ⁴	3.05±0.11×10 ³	2.35±0.18×10 ³	3.35±0.11×10 ⁴	1.18±0.04×10 ⁴	9.67±1.55×10 ³				
			Pigmented	2.67±0.35×10 ³	8.67±3.00×10 ³	1.63±0.42×10 ²	8.33±0.00×10 ¹	2.37±0.13×10 ⁴	2.93±0.75×10 ³	5.03±2.10×10 ³				
			AAP	7.41×10 ²	1.93×10 ³	2.04×10 ¹	0	3.38×10 ³	1.30×10 ³	3.36×10 ³				
		RO _r	Total	1.17±0.30×10 ²	5.67±2.50×10 ¹	0	0	6.67±5.00×10 ⁰	6.67±0.00×10 ⁰	0				
			Pigmented	7.00±0.50×10 ¹	2.00±3.00×10 ¹	0	0	3.33±0.00×10 ⁰	6.67±0.00×10 ⁰	0				
			AAP	0	6.67×10 ⁰	0	0	0	0	0				
		Potato	Total	8.67±2.50×10 ²	8.33±2.00×10 ³	1.00±0.36×10 ³	2.40±0.10×10 ³	1.03±0.15×10 ³	1.10±0.10×10 ³	5.67±2.50×10 ²				
			Pigmented	7.80±0.40×10 ³	1.14±0.05×10 ⁵	4.63±0.79×10 ³	3.67±0.50×10 ³	3.47±0.25×10 ³	1.80±0.15×10 ³	9.33±1.50×10 ²				
			AAP	1.49×10 ³	4.97×10 ³	1.05×10 ³	2.44×10 ²	2.04×10 ²	4.29×10 ²	5.33×10 ²				
		BG-11	Total	2.29±0.09×10 ⁴	1.55±0.08×10 ⁵	6.00±0.00×10 ³	2.92±0.20×10 ³	1.38±0.65×10 ⁴	1.21±0.13×10 ⁴	6.83±0.25×10 ³				
			AOP	2.30±1.80×10 ²	2.00±0.50×10 ²	2.03±2.74×10 ²	2.77±0.15×10 ²	0	0	0				
			AAP	2.30±1.80×10 ²	2.00±0.50×10 ²	2.03±2.74×10 ²	2.77±0.15×10 ²	0	0	0				
		PNSm	Total	1.10±0.45×10 ²	1.41±0.54×10 ³	4.00±1.63×10 ¹	0	9.33±0.50×10 ¹	5.67±0.50×10 ¹	3.37±0.10×10 ²				
			AnAnP	0	2.70±0.35×10 ²	0	0	0	0	0				
			AAP	0	2.70±0.35×10 ²	1.88×10 ²	1.21×10 ²	1.63×10 ¹	1.38×10 ²	0				
		Summer	RO	Total	1.45±0.00×10 ⁴	5.15±0.25×10 ⁵	5.37±0.13×10 ³	4.92±0.73×10 ³	7.80±0.95×10 ²	3.20±0.65×10 ³	8.03±0.25×10 ²			
				Pigmented	4.50±0.00×10 ³	1.55±0.15×10 ⁵	4.50±0.08×10 ³	4.11±0.61×10 ³	1.63±0.05×10 ²	9.67±1.00×10 ²	4.40±0.30×10 ²			
				AAP	0	0	1.88×10 ²	1.21×10 ²	1.63×10 ¹	1.38×10 ²	0			
			OM	Total	1.77±0.65×10 ⁴	1.40±0.11×10 ⁶	5.60±1.06×10 ³	5.44±0.46×10 ³	1.21±0.00×10 ³	4.47±0.15×10 ³	1.47±0.01×10 ³			
				Pigmented	2.43±0.00×10 ³	2.33±0.20×10 ⁵	4.53±1.10×10 ³	4.37±0.29×10 ³	2.10±0.00×10 ²	1.10±0.40×10 ³	5.17±0.70×10 ²			
				AAP	8.39×10 ¹	1.46×10 ⁴	1.36×10 ³	0	2.10×10 ¹	8.80×10 ¹	0			
	RO _r		Total	9.33±2.00×10 ¹	1.40±0.65×10 ³	1.37±0.24×10 ²	1.47±0.10×10 ²	2.67±0.50×10 ¹	3.67±0.00×10 ¹	4.67±3.00×10 ¹				
			Pigmented	8.33±0.50×10 ¹	1.13±0.40×10 ³	1.23±0.13×10 ²	1.30±0.00×10 ²	1.67±1.50×10 ¹	3.67±0.00×10 ¹	4.33±3.00×10 ¹				
			AAP	0	0	0	0	0	0	0				
	Potato		Total	3.90±0.95×10 ³	7.83±3.10×10 ⁴	6.30±0.78×10 ²	6.10±0.40×10 ³	6.57±0.05×10 ²	1.16±0.09×10 ³	5.63±1.05×10 ²				
			Pigmented	1.97±0.45×10 ³	2.37±1.00×10 ⁴	5.13±0.74×10 ³	5.23±0.50×10 ³	1.07±0.05×10 ²	3.80±0.45×10 ²	3.23±0.25×10 ²				
			AAP	1.64×10 ²	1.18×10 ³	0	0	9.28×10 ⁰	2.38×10 ¹	2.81×10 ¹				
	BG-11		Total	4.30±1.35×10 ⁴	1.83±0.30×10 ⁶	4.27±0.13×10 ³	7.40±1.00×10 ³	1.29±0.21×10 ⁴	1.39±0.08×10 ³	1.66±0.14×10 ³				
			AOP	1.93±0.15×10 ³	0	9.33±2.05×10 ¹	2.00±0.00×10 ¹	8.33±6.00×10 ²	0	0				
			AAP	1.93±0.15×10 ³	0	9.33±2.05×10 ¹	2.00±0.00×10 ¹	8.33±6.00×10 ²	0	0				
	PNSm		Total	9.30±0.15×10 ²	1.03±0.55×10 ⁴	2.67±0.47×10 ¹	2.67±0.5×10 ¹	1.30±0.45×10 ²	5.00±0.00×10 ¹	3.00±1.00×10 ¹				
			AnAnP	3.33±5.00×10 ⁰	1.67±0.50×10 ¹	0	0	0	0	0				
			AAP	3.33±5.00×10 ⁰	1.67±0.50×10 ¹	0	0	0	0	0				
	Fall		RO	Total	9.30±0.30×10 ³	2.50±0.14×10 ⁵	1.03±0.56×10 ⁴	5.57±0.40×10 ³	8.73±2.45×10 ³	1.01±0.22×10 ³	3.87±0.25×10 ³			
				Pigmented	2.93±0.10×10 ³	5.10±0.55×10 ⁴	5.70±4.05×10 ³	2.83±0.15×10 ³	2.30±0.90×10 ³	1.47±0.05×10 ²	1.10±0.50×10 ³			
				AAP	7.04×10 ²	5.80×10 ³	0	0	0	0	0			
			OM	Total	1.41±0.14×10 ⁴	9.87±2.40×10 ⁴	2.59±0.26×10 ⁴	7.73±0.28×10 ³	5.60±0.10×10 ⁴	6.23±1.50×10 ³	1.28±0.08×10 ⁴			
				Pigmented	2.00±0.70×10 ³	3.67±0.75×10 ⁴	5.87±0.42×10 ³	3.43±0.90×10 ³	1.13±0.45×10 ⁴	8.67±4.50×10 ²	3.70±1.85×10 ³			
				AAP	0	9.91×10 ²	0	0	0	0	0			
		RO _r	Total	2.70±0.30×10 ²	4.17±0.65×10 ³	1.33±0.96×10 ²	1.67±0.15×10 ²	2.00±0.00×10 ¹	0	0				
			Pigmented	2.70±0.30×10 ²	4.03±0.55×10 ³	1.33±0.96×10 ²	1.63±0.20×10 ²	2.00±0.00×10 ¹	0	0				
			AAP	0	0	0	0	0	0	0				
		Potato	Total	4.27±0.50×10 ³	1.89±0.15×10 ⁴	5.10±0.99×10 ³	1.06±0.15×10 ³	7.53±1.10×10 ³	8.77±0.20×10 ²	1.29±0.13×10 ³				
			Pigmented	1.97±0.35×10 ³	5.87±0.40×10 ³	2.70±0.74×10 ³	4.77±0.70×10 ²	1.40±0.65×10 ³	1.40±0.10×10 ²	2.40±0.40×10 ²				
			AAP	8.55×10 ¹	4.51×10 ²	0	0	0	0	0				
		BG-11	Total	4.13±0.40×10 ⁴	1.26±0.08×10 ⁵	2.90±0.08×10 ⁴	8.17±0.75×10 ²	1.67±0.90×10 ²	1.30±0.15×10 ²	1.38±1.08×10 ⁴				
			AOP	7.33±0.00×10 ²	1.70±0.05×10 ³	2.67±0.09×10 ¹	1.00±0.50×10 ¹	0	3.3±0.00×10 ⁰	1.33±1.00×10 ¹				
			AAP	7.33±0.00×10 ²	1.70±0.05×10 ³	2.67±0.09×10 ¹	1.00±0.50×10 ¹	0	3.3±0.00×10 ⁰	1.33±1.00×10 ¹				
		PNSm	Total	8.67±1.50×10 ¹	1.52±1.73×10 ⁴	1.33±0.47×10 ¹	1.33±0.50×10 ¹	1.20±0.30×10 ²	1.67±1.50×10 ¹	1.67±1.50×10 ¹				
	AnAnP		0	2.67±0.00×10 ¹	0	0	0	0	0					
	2018	Winter	Alternative Sites			W10		W1		W9		W6		W8
RO			Total	N/A	N/A	8.20±0.60×10 ²	4.03±0.21×10 ²	8.63±0.90×10 ²	1.09±0.04×10 ³	1.53±0.05×10 ²				
			Pigmented	N/A	N/A	4.43±2.15×10 ²	2.47±0.47×10 ²	5.57±0.05×10 ²	6.80±0.10×10 ²	7.00±0.5×10 ¹				
			AAP	N/A	N/A	0	0	0	0	1.48×10 ¹				
OM			Total	N/A	N/A	1.54±0.06×10 ³	6.65±1.15×10 ²	1.64±0.00×10 ³	1.52±0.19×10 ³	3.70±0.55×10 ²				
			Pigmented	N/A	N/A	8.50±0.15×10 ²	3.15±0.15×10 ²	9.20±0.00×10 ²	3.00±0.60×10 ²	8.00±1.50×10 ¹				
			AAP	N/A	N/A	0	0	0	0	0				
RO _r			Total	N/A	N/A	6.67±0.50×10 ¹	6.67±9.43×10 ⁰	7.00±1.50×10 ¹	5.00±1.00×10 ¹	6.67±5.00×10 ⁰				
			Pigmented	N/A	N/A	4.67±0.00×10 ¹	6.67±9.43×10 ⁰	6.67±1.50×10 ¹	3.00±4.00×10 ¹	6.67±5.00×10 ⁰				
			AAP	N/A	N/A	0	0	0	0	0				
Potato			Total	N/A	N/A	7.90±0.20×10 ²	N/A	7.75±1.15×10 ²	N/A	1.77±0.45×10 ²				
			Pigmented	N/A	N/A	6.27±0.50×10 ²	N/A	7.05±1.35×10 ²	N/A	7.00±2.50×10 ¹				
			AAP	N/A	N/A	0	0	0	0	0				
BG-11			Total	N/A	N/A	1.79±0.03×10 ³	4.60±4.81×10 ²	1.49±0.32×10 ³	1.85±1.22×10 ³	7.27±2.20×10 ²				
			AOP	N/A	N/A	0	0	0	0	0				
			AAP	N/A	N/A	0	0	0.67±1.00×10 ¹	3.00±0.50×10 ¹	3.33±5.00×10 ⁰				
PNSm			Total	N/A	N/A	0	0	0	0	0				
			AnAnP	N/A	N/A	0	0	0	0	0				

Table 2.4. Continued (6/7).

				5	6	7	8	9	10		
Year	Season	Medium		liquid	sediment	liquid	liquid	liquid	liquid	liquid	
2018	Spring	RO	Total	5.93±0.90×10 ²	5.07±0.30×10 ⁴	9.93±0.81×10 ²	3.63±1.10×10 ³	9.27±1.50×10 ²	1.10±0.24×10 ³	7.00±0.00×10 ²	
			Pigmented	2.50±0.20×10 ³	1.60±0.15×10 ⁴	3.67±0.21×10 ²	1.97±0.60×10 ³	2.97±0.25×10 ²	5.47±0.55×10 ²	2.50±0.00×10 ²	
		AAP	3.91×10 ²	2.46×10 ³	0	5.46×10 ¹	0	4.56×10 ¹	1.67×10 ¹		
			OM	Total	1.08±0.50×10 ³	1.22±0.43×10 ⁵	3.75±0.26×10 ³	9.30±0.15×10 ³	3.77±0.90×10 ³	6.37±1.10×10 ³	6.67±0.45×10 ³
		Pigmented		1.77±0.50×10 ³	1.77±1.15×10 ⁴	4.47±0.54×10 ²	1.70±0.10×10 ³	1.13±0.30×10 ³	7.00±1.00×10 ²	6.63±2.00×10 ²	
		AAP	0	0	0	5.15×10 ¹	0	0	4.87×10 ¹		
			RO _r	Total	1.47±0.10×10 ²	2.77±0.30×10 ³	1.67±0.94×10 ¹	3.33±2.00×10 ¹	2.00±1.50×10 ¹	0	0.67±1.00×10 ¹
		Pigmented		1.10±0.15×10 ²	2.50±0.10×10 ³	1.33±1.25×10 ¹	3.33±2.00×10 ¹	2.00±1.50×10 ¹	0	0.67±1.00×10 ¹	
		AAP	0	8.33×10 ²	0	0	0	0	0	0	
			Potato	Total	1.09±0.23×10 ³	7.93±2.65×10 ³	2.43±0.61×10 ²	3.07±0.30×10 ²	2.07±0.25×10 ²	3.00±1.50×10 ¹	2.33±1.00×10 ¹
		Pigmented		5.13±2.40×10 ²	1.20±0.40×10 ³	1.30±0.36×10 ²	1.87±0.20×10 ²	2.33±1.50×10 ¹	1.67±1.00×10 ¹	1.00±0.50×10 ¹	
		AAP	1.12×10 ²	2.18×10 ²	0	0	0	0	0	0	
			BG-11	Total	1.45±0.08×10 ⁴	4.47±0.15×10 ⁵	2.15±0.16×10 ³	1.97±0.05×10 ³	1.59±0.012×10 ³	5.23±1.15×10 ³	1.67±0.50×10 ²
		AOP		5.33±0.50×10 ¹	3.33±0.00×10 ²	1.53±0.18×10 ³	9.00±5.00×10 ¹	1.90±0.65×10 ²	0	0	
		PNSm	Total	3.33±2.50×10 ¹	1.16±0.01×10 ³	5.87±1.66×10 ²	3.00±1.00×10 ¹	9.33±4.50×10 ¹	2.67±0.00×10 ¹	5.00±1.50×10 ¹	
			AnAnP	0	0	0	0	0	0	0	
		Summer	RO	Total	1.55±0.12×10 ⁴	5.67±0.40×10 ⁴	8.67±0.94×10 ¹	4.50±0.00×10 ¹	1.70±0.10×10 ²	3.15±0.00×10 ²	2.17±0.00×10 ³
				Pigmented	8.23±2.00×10 ³	2.87±1.00×10 ⁴	0	5.00±0.00×10 ⁰	2.67±0.00×10 ¹	1.45±0.00×10 ²	3.30±0.00×10 ²
			AAP	0	0	0	0	0	0	0	
				OM	Total	2.34±0.05×10 ⁴	1.45±0.21×10 ⁵	3.27±0.85×10 ²	8.77±1.40×10 ²	3.97±0.30×10 ²	1.25±0.13×10 ³
	Pigmented		6.13±0.90×10 ³		3.00±1.00×10 ⁴	5.33±2.87×10 ¹	6.67±0.50×10 ¹	1.00±0.35×10 ²	1.77±0.10×10 ²	4.50±2.80×10 ²	
	AAP		0	0	0	0	0	0	0		
			RO _r	Total	1.08±0.02×10 ³	1.16±0.04×10 ⁴	3.33±4.71×10 ⁰	3.30±5.00×10 ⁰	6.67±0.00×10 ⁰	1.33±1.00×10 ¹	2.00±2.00×10 ¹
	Pigmented			9.37±0.60×10 ²	9.77±1.10×10 ³	3.33±4.71×10 ⁰	3.33±5.00×10 ⁰	6.67±0.00×10 ⁰	0	2.00±2.00×10 ¹	
	AAP		0	0	0	0	0	0	0		
			Potato	Total	3.55±0.84×10 ³	8.73±1.35×10 ³	3.33±1.70×10 ¹	2.33±0.50×10 ¹	3.67±1.00×10 ¹	1.33±1.00×10 ¹	1.49±0.01×10 ³
	Pigmented			2.89±0.71×10 ³	4.80±0.75×10 ²	0	0	3.33±5.00×10 ⁰	0	1.20±0.15×10 ²	
	AAP		0	0	0	0	0	0	0		
			BG-11	Total	1.33±2.00×10 ⁷	N/A	9.00±4.20×10 ²	2.47±2.45×10 ³	1.77±1.00×10 ²	8.67±1.50×10 ¹	1.68±0.05×10 ³
	AOP			2.77±0.25×10 ²	1.47±0.25×10 ³	0	2.00±0.50×10 ¹	0	0	0	
	PNSm		Total	6.53±0.35×10 ²	4.83±0.30×10 ²	0	6.67±5.00×10 ⁰	6.67±5.00×10 ⁰	0.67±1.00×10 ¹	1.61±0.29×10 ³	
			AnAnP	0	0	0	0	0	0	0	
	Fall		RO	Total	3.87±1.15×10 ³	2.27±0.10×10 ⁴	5.07±0.63×10 ³	N/A	1.18±0.06×10 ⁴	2.34±0.06×10 ³	N/A
				Pigmented	1.33±0.10×10 ³	4.33±1.50×10 ³	4.03±0.69×10 ³	N/A	4.00±0.50×10 ³	6.47±0.00×10 ²	N/A
			AAP	1.98×10 ²	0	8.58×10 ¹	N/A	0	0	N/A	
				OM	Total	8.07±1.20×10 ³	7.53±0.00×10 ⁴	9.57±1.18×10 ³	N/A	5.73±0.90×10 ⁴	1.05±0.17×10 ⁴
		Pigmented	2.03±0.15×10 ³		1.10±0.00×10 ⁴	3.00±2.41×10 ³	N/A	1.80±1.20×10 ⁴	3.03±0.00×10 ³	N/A	
		AAP	0	0	0	N/A	3.91×10 ²	0	N/A		
			RO _r	Total	3.43±0.65×10 ²	2.68±0.51×10 ³	1.84±0.36×10 ³	N/A	2.33±0.50×10 ¹	3.33±1.50×10 ¹	N/A
		Pigmented		3.30±0.65×10 ²	1.51±0.32×10 ³	1.84±0.36×10 ³	N/A	2.33±0.50×10 ¹	3.33±1.50×10 ¹	N/A	
AAP		0	0	0	N/A	0	0	N/A			
		Potato	Total	2.03±0.25×10 ³	1.05±0.27×10 ⁴	3.60±0.29×10 ³	N/A	6.83±0.15×10 ²	7.17±0.00×10 ²	N/A	
Pigmented			7.03±0.30×10 ²	1.47±0.35×10 ³	3.03±0.37×10 ³	N/A	8.67±2.00×10 ¹	2.83±0.40×10 ²	N/A		
AAP		0	0	0	N/A	6.19×10 ⁰	0	N/A			
		BG-11	Total	3.25±0.65×10 ⁴	2.02±0.22×10 ⁵	2.09±0.20×10 ⁴	N/A	N/A	N/A	N/A	
AOP			1.87±0.35×10 ³	9.60±0.00×10 ³	1.67±0.47×10 ¹	N/A	4.00±0.00×10 ¹	2.00±0.00×10 ¹	N/A		
PNSm		Total	1.47±0.00×10 ²	2.54±0.13×10 ³	5.67±0.47×10 ¹	N/A	2.00±0.50×10 ¹	1.00±1.00×10 ¹	N/A		
		AnAnP	0	3.33±5.00×10 ⁰	2.00±1.41×10 ¹	N/A	0	0	N/A		
2019		Winter	Alternative Sites				W10	W1	W9	W6	W8
				RO	Total	N/A	N/A	3.83±0.80×10 ²	3.30±1.27×10 ²	5.10±0.10×10 ²	3.00±0.40×10 ²
			Pigmented	N/A	N/A	1.50±0.60×10 ²	1.37±0.97×10 ²	1.50±0.05×10 ²	1.20±0.15×10 ²	1.47±0.05×10 ²	
				AAP	N/A	N/A	0	0	0	0	0
	OM		Total		N/A	N/A	4.90±0.20×10 ²	2.83±1.23×10 ²	6.27±0.75×10 ²	2.10±0.05×10 ²	3.93±0.15×10 ²
			Pigmented	N/A	N/A	1.33±0.35×10 ²	2.00±1.63×10 ¹	1.70±0.35×10 ²	2.67±0.05×10 ¹	8.67±1.00×10 ¹	
	AAP		N/A	N/A	0	0	0	0	0		
			RO _r	Total	N/A	N/A	1.50±0.50×10 ¹	0	3.33±5.00×10 ⁰	1.67±2.50×10 ¹	3.33±5.00×10 ⁰
	Pigmented			N/A	N/A	1.50±0.50×10 ¹	0	3.33±5.00×10 ⁰	0.66±1.00×10 ¹	3.33±5.00×10 ⁰	
	AAP		N/A	N/A	0	0	0	0	0		
			Potato	Total	N/A	N/A	3.20±1.20×10 ²	2.37±1.03×10 ²	3.90±0.20×10 ²	1.27±0.20×10 ²	3.10±0.40×10 ²
	Pigmented			N/A	N/A	1.50±0.50×10 ²	1.27±0.56×10 ²	2.10±0.20×10 ²	5.33±2.00×10 ¹	1.80±0.00×10 ²	
	AAP		N/A	N/A	0	0	0	0	0		
			R2A	Total	N/A	N/A	N/A	5.40±2.79×10 ²	N/A	5.40±0.90×10 ²	N/A
	Pigmented			N/A	N/A	N/A	2.50±1.10×10 ²	N/A	2.50±0.60×10 ²	N/A	
	AAP		N/A	N/A	N/A	0	N/A	0	N/A	N/A	
			BG-11	Total	N/A	N/A	4.63±0.10×10 ²	4.77±0.73×10 ²	5.97±0.25×10 ²	2.08±0.79×10 ³	5.43±0.05×10 ²
	AOP			N/A	N/A	0	3.33±4.71×10 ⁰	0	0	0	
	PNSm		Total	N/A	N/A	0	0	0	0	0	
			AnAnP	N/A	N/A	0	0	0	0	0	

Table 2.4. Continued (7/7).

				5	6	7	8	9	10			
Year	Season	Medium		liquid	sediment	liquid	liquid	liquid	liquid			
2019	Spring	RO	Total	4.80±0.35×10 ⁴	9.33±0.30×10 ³	1.51±0.07×10 ³	7.60±0.45×10 ³	3.56±1.28×10 ³	9.77±1.60×10 ³	5.87±0.05×10 ²		
			Pigmented	1.17±0.20×10 ⁴	2.70±0.50×10 ³	1.29±0.20×10 ⁵	7.27±0.40×10 ³	2.81±0.87×10 ³	4.47±0.25×10 ³	4.30±0.85×10 ²		
			AAP	7.07×10 ²	0	2.52×10 ³	8.38×10 ²	1.59×10 ²	5.32×10 ²	1.72×10 ¹		
		OM	Total	7.73±0.25×10 ⁴	3.53±0.50×10 ⁴	2.30±0.78×10 ⁴	1.16±0.04×10 ⁴	2.14±0.08×10 ⁴	1.18±0.16×10 ⁴	5.13±0.65×10 ³		
			Pigmented	4.00±2.50×10 ³	7.67±3.00×10 ³	1.23±0.39×10 ⁴	8.20±0.50×10 ³	9.70±0.60×10 ³	3.80±0.45×10 ³	6.60±0.10×10 ²		
			AAP	2.22×10 ²	1.21×10 ³	0	5.13×10 ²	1.01×10 ³	7.04×10 ²	0		
		RO _r	Total	2.20±0.25×10 ³	1.79±0.20×10 ³	6.07±0.29×10 ³	2.24±0.16×10 ³	1.97±0.25×10 ³	9.03±0.05×10 ²	8.67±2.00×10 ¹		
			Pigmented	2.13±0.18×10 ³	1.58±0.42×10 ³	6.07±0.29×10 ³	2.20±0.13×10 ³	1.93±0.26×10 ³	8.47±0.00×10 ²	8.67±2.00×10 ¹		
			AAP	0	5.91×10 ²	0	0	0	0	0		
		Potato	Total	2.93±0.85×10 ⁴	5.37±0.05×10 ³	1.00±0.13×10 ⁴	3.67±0.25×10 ³	1.00±0.00×10 ⁴	2.13±0.03×10 ³	1.21±0.13×10 ³		
			Pigmented	1.30±0.45×10 ⁴	1.73±0.20×10 ³	8.77±0.87×10 ³	3.43±0.30×10 ³	1.00±0.00×10 ⁴	6.33±9.50×10 ²	7.80±1.45×10 ²		
			AAP	7.65×10 ²	3.47×10 ²	1.95×10 ³	4.29×10 ²	0	3.17×10 ¹	4.88×10 ¹		
		R2A	Total	8.40±2.45×10 ⁴	3.67±0.25×10 ⁴	1.91±0.10×10 ⁴	N/A	1.49±0.01×10 ⁴	1.11±0.07×10 ⁴	6.50±1.05×10 ³		
			Pigmented	2.33±0.70×10 ⁴	1.23±0.20×10 ⁴	9.13±6.46×10 ³	N/A	8.40±0.50×10 ³	5.43±0.20×10 ³	2.80±0.45×10 ³		
			AAP	0	3.36×10 ³	4.35×10 ²	N/A	1.80×10 ³	0	1.08×10 ²		
		BG-11	Total	8.33±0.85×10 ⁴	3.09±0.44×10 ⁴	1.78±0.26×10 ⁴	5.35±0.00×10 ⁴	1.78±0.07×10 ⁴	1.29±0.26×10 ⁴	2.41±0.45×10 ⁴		
			AOP	0	3.33±5.00×10 ⁰	1.33±1.25×10 ¹	0	0	6.67±1.00×10 ¹	0		
			PNSm	Total	9.37±0.25×10 ³	1.05±0.01×10 ⁴	1.33±1.25×10 ¹	2.33±2.00×10 ¹	3.33±0.00×10 ⁰	1.33±0.05×10 ¹	0.67±1.00×10 ¹	
		Summer	RO	AnAnP	0	0	0	0	0	0	0	
				Total	7.40±0.10×10 ²	5.45±0.00×10 ⁵	2.85±0.06×10 ³	2.83±0.03×10 ³	2.63±0.25×10 ³	3.24±1.07×10 ³	7.77±1.05×10 ³	
				Pigmented	1.13±0.20×10 ²	1.30±0.00×10 ⁵	1.23±0.06×10 ³	1.08±0.14×10 ³	8.33±2.50×10 ²	6.47±1.30×10 ²	4.13±0.20×10 ³	
	OM		AAP	0	1.34×10 ⁴	0	0	0	0	1.80×10 ²		
			Total	1.85±0.00×10 ³	6.10±1.85×10 ⁵	4.50±0.80×10 ³	5.33±0.75×10 ³	1.83±0.18×10 ⁴	5.30±0.45×10 ³	1.41±0.00×10 ⁴		
			Pigmented	2.10±0.00×10 ²	8.00±1.00×10 ⁴	2.33±1.25×10 ²	2.67±0.00×10 ²	2.20±0.05×10 ³	1.77±0.00×10 ³	1.65±0.00×10 ³		
	RO _r		AAP	0	3.08×10 ³	8.33×10 ⁰	9.52×10 ⁰	0	0	1.10×10 ²		
			Total	4.00±0.50×10 ¹	3.77±1.20×10 ⁴	3.50±1.61×10 ²	7.33±5.00×10 ¹	4.33±1.00×10 ¹	1.00±0.10×10 ²	3.00±0.50×10 ¹		
			Pigmented	3.33±0.00×10 ¹	3.60±1.05×10 ⁴	3.50±1.61×10 ²	7.33±5.00×10 ¹	4.33±1.00×10 ¹	1.00±0.10×10 ²	3.00±0.50×10 ¹		
	Potato		AAP	4.76×10 ⁰	7.20×10 ³	7.00×10 ¹	1.47×10 ¹	7.88×10 ⁰	0	0		
			Total	4.07±0.75×10 ²	4.23±0.30×10 ⁴	9.60±0.78×10 ³	1.35±0.06×10 ³	7.07±0.35×10 ³	8.00±0.00×10 ³	2.40±0.00×10 ³		
			Pigmented	9.67±0.50×10 ¹	1.77±0.10×10 ⁴	2.03±0.24×10 ³	8.13±0.25×10 ²	3.33±0.15×10 ³	1.20±0.00×10 ³	1.91±0.00×10 ³		
	R2A		AAP	1.76×10 ¹	2.52×10 ³	1.45×10 ²	4.78×10 ¹	1.52×10 ²	0	1.12×10 ²		
			Total	1.54±0.36×10 ³	1.90±0.29×10 ⁵	1.45±0.12×10 ³	1.86±0.08×10 ³	5.43±0.90×10 ³	1.10±0.26×10 ⁴	5.57±1.20×10 ³		
			Pigmented	2.27±0.30×10 ²	2.57±0.25×10 ⁴	7.67±0.63×10 ²	7.53±0.20×10 ²	1.53±0.20×10 ³	1.47±0.30×10 ³	2.77±0.30×10 ³		
	BG-11		AAP	0	2.14×10 ³	3.65×10 ¹	3.42×10 ¹	1.92×10 ²	0	3.95×10 ²		
			Total	3.07±0.10×10 ⁴	1.22±0.01×10 ⁶	7.73±0.46×10 ³	4.53±0.05×10 ³	5.43±1.85×10 ³	1.22±0.71×10 ⁴	6.23±0.10×10 ³		
			AOP	1.27±0.13×10 ³	2.97±0.03×10 ⁴	1.67±0.47×10 ¹	2.33±0.50×10 ¹	6.33±2.50×10 ¹	5.37±1.55×10 ²	2.40±1.20×10 ²		
	PNSm		Total	4.53±0.10×10 ³	1.12±0.06×10 ⁴	8.87±2.19×10 ²	1.23±0.15×10 ³	3.33±0.00×10 ¹	7.90±0.10×10 ²	3.67±1.50×10 ¹		
			AnAnP	7.33±2.00×10 ²	0	7.67±2.96×10 ²	5.40±0.40×10 ²	0	0	0		
			Total	2.06±0.11×10 ⁴	1.72±0.47×10 ⁵	1.47±0.03×10 ⁴	1.78±0.07×10 ³	1.66±0.14×10 ⁴	5.80±1.70×10 ³	6.13±0.35×10 ⁴		
	Fall	RO	Pigmented	4.17±0.35×10 ³	5.53±0.80×10 ⁴	1.03±0.05×10 ⁴	9.47±0.40×10 ²	8.57±1.40×10 ³	3.00±0.60×10 ³	1.27±0.95×10 ⁴		
			AAP	0	0	3.21×10 ²	0	0	0	2.88×10 ²		
			Total	5.73±0.15×10 ⁴	9.07±0.50×10 ⁵	1.80±0.10×10 ⁴	7.97±3.50×10 ³	1.62±0.33×10 ⁴	9.17±0.15×10 ³	4.70±3.80×10 ⁴		
		OM	Pigmented	7.00±1.00×10 ³	1.17±0.05×10 ⁵	1.25±0.15×10 ⁴	1.57±0.15×10 ³	5.60±1.20×10 ³	2.43±0.15×10 ³	7.33±3.50×10 ³		
			AAP	0	5.83×10 ³	2.27×10 ³	0	0	0	8.84×10 ¹		
			Total	3.23±0.65×10 ³	5.03±0.80×10 ⁴	2.60±1.10×10 ³	3.73±0.25×10 ²	2.47±0.40×10 ²	1.13±0.71×10 ³	7.07±0.85×10 ²		
		RO _r	Pigmented	3.00±0.60×10 ³	4.97±0.70×10 ⁴	2.60±1.10×10 ³	3.63±0.15×10 ²	2.40±0.35×10 ²	1.11±0.73×10 ³	7.07±0.85×10 ²		
			AAP	9.23×10 ²	0	0	0	1.26×10 ¹	0	1.09×10 ²		
			Total	5.73±2.65×10 ³	5.57±0.85×10 ⁴	1.43±0.07×10 ⁴	1.44±0.12×10 ³	8.47±0.65×10 ²	2.30±0.21×10 ³	3.90±0.50×10 ³		
		Potato	Pigmented	2.47±0.20×10 ³	1.83±0.55×10 ⁴	1.03±0.08×10 ⁴	8.47±0.50×10 ²	5.00±0.90×10 ²	1.17±0.32×10 ³	1.60±0.25×10 ³		
			AAP	0	2.62×10 ³	0	8.06×10 ¹	0	0	0		
			Total	1.60±0.15×10 ⁴	2.25±0.22×10 ⁵	2.60±1.10×10 ³	8.20±0.15×10 ³	7.63±0.70×10 ³	4.43±0.50×10 ³	1.61±0.30×10 ⁴		
		R2A	Pigmented	2.97±1.25×10 ³	6.03±1.05×10 ⁴	2.60±1.10×10 ³	1.63±0.10×10 ³	1.93±0.10×10 ³	2.23±0.50×10 ³	2.23±0.85×10 ³		
			AAP	0	1.51×10 ⁴	1.04×10 ²	0	0	0	9.71×10 ¹		
			Total	7.33±1.35×10 ⁴	9.67±1.60×10 ⁵	4.23±0.13×10 ⁴	5.37±1.90×10 ²	1.86±0.10×10 ⁴	2.17±0.15×10 ⁴	6.57±5.00×10 ⁴		
		BG-11	AOP	1.10±0.30×10 ²	4.50±0.00×10 ³	0	0	6.67±0.00×10 ⁰	8.67±0.50×10 ¹	2.33±0.40×10 ²		
			Total	3.17±0.15×10 ²	4.63±1.33×10 ³	1.47±0.41×10 ²	6.33±4.50×10 ¹	3.33±5.00×10 ⁰	1.33±1.00×10 ¹	2.67±0.50×10 ¹		
			AnAnP	0	0	0	0	0	0	0		
		2020	Winter	Alternative Sites	Total	N/A	N/A	8.67±1.00×10 ¹	1.93±0.31×10 ²	2.33±1.00×10 ¹	1.20±0.05×10 ²	9.67±2.00×10 ¹
					Pigmented	N/A	N/A	0	5.00±2.16×10 ¹	1.33±1.00×10 ¹	3.33±1.50×10 ¹	2.33±0.00×10 ¹
					AAP	N/A	N/A	0	0	0	2.67×10 ⁰	0
	OM			Total	N/A	N/A	6.53±0.35×10 ²	7.00±0.00×10 ¹	6.00±0.00×10 ¹	2.70±0.55×10 ²	3.00±0.50×10 ¹	
				Pigmented	N/A	N/A	0	2.33±1.70×10 ¹	0	2.67±2.50×10 ¹	0	
				AAP	N/A	N/A	0	0	0	0	0	
RO _r	Total			N/A	N/A	6.67±0.00×10 ⁰	6.33±1.25×10 ¹	4.00±1.00×10 ¹	0	1.00±0.50×10 ¹		
	Pigmented			N/A	N/A	0	6.67±4.71×10 ⁰	0	3.00±1.00×10 ¹	1.00±0.50×10 ¹		
	AAP			N/A	N/A	0	0	0	0	0		
Potato	Total			N/A	N/A	6.67±0.00×10 ⁰	6.00±1.41×10 ¹	0	3.00±1.50×10 ¹	2.33±1.00×10 ¹		
	Pigmented			N/A	N/A	0	1.00±0.82×10 ¹	0	3.33±0.00×10 ⁰	0		
	AAP			N/A	N/A	0	0	0	0	0		
R2A	Total	N/A	N/A	2.90±1.10×10 ²	5.90±0.71×10 ²	1.27±0.20×10 ²	5.67±2.5×10 ¹	1.27±0.05×10 ²				
	Pigmented	N/A	N/A	5.67±2.00×10 ¹	3.67±1.00×10 ¹	3.67±1.00×10 ¹	6.67±0.00×10 ⁰	2.00±1.00×10 ¹				
	AAP	N/A	N/A	0	0	0	0	1.13×10 ¹				
BG-11	Total	N/A	N/A	1.28±0.31×10 ³	2.70±0.29×10 ²	4.17±0.55×10 ²	4.00±0.00×10 ¹	6.00±3.00×10 ¹				
	AOP	N/A	N/A	1.33±0.50×10 ¹	3.67±2.49×10 ¹	0	3.33±5.00×10 ⁰	0				
	PNSm	Total	N/A	N/A	0	0	6.00±0.00×10 ¹	0	0			
		AnAnP	N/A	N/A	0	0	0	0	0			

2.4.4. Microbial community eDNA sequencing

Environmental DNA (eDNA) was extracted from liquid or sediment samples using a QIAGEN DNeasy® PowerWater® Kit with minor modifications, and bacterial 16S V4 rRNA genes were sequenced by MiSeq (Illumina) with primer set 515F, 806R. Community structures were computationally analyzed with Qiime2 11.3, a Python based framework (McKinney, 2010; Boyen *et al.*, 2019), and BIOM formatting (McDonald *et al.*, 2012). Reads were joined via paired-ends, trimmed to 292 bp (kept > 99%), then deblurred (Amir *et al.*, 2017) and denoised (Bokulich *et al.*, 2013), prior to *de novo* clustering (binning) sequences by 99% identity. Operational taxonomic unit (OTU) alignment were applied using 99% similarity to type species (Price *et al.*, 2010; Rognes *et al.*, 2016) to the Silva132 database (Quast *et al.*, 2012), with a final data set that included “borderline-chimeras”. Phenetic identities were aligned as feature tables (Weiss *et al.*, 2017) at all hierarchical levels of the phylogenetic tree (Stackebrandt and Goodfellow, 1991; Katoh and Standley, 2013). Both α - and β -diversity were statistically quantified, and graphed as boxplots or principle coordinate analysis (PCoA) (Faith *et al.*, 1987; Vázquez-Baeza *et al.*, 2017). Qiime2 project code text file describing entire pipeline and final output in both Qiime2 and BIOM formats were provided as Supplemental Materials. The AOP, AAP, and AnAnP proportions of the sequenced communities were analyzed by grouping all representative OTUs to known type species, Tables 1.1 and 1.2.

2.5. Results

2.5.1. Environmental characteristics

Seasonal observations of sites over 4 years (Table 2.1) revealed trends in physicochemical environmental factors (Fig. 2.2). Daylight was the longest during the spring with marginal variation (due to sampling times), shortening into the fall (Fig. 2.2). In comparison, lake water did not warm up until the peak of summer, decreasing to near 0°C just below the ice surface at limnetic zones during winter. Littoral areas reached an average high of 21.5°C, with some sites up to 26.0°C, while limnetic regions had a moderate peak around 20.2°C. Littoral warmed faster in the spring and summer, but was also colder than limnetic in the fall, which represented heat retention of open water. General trends in pH also followed seasonal changes, with winter limnetic zones being the most neutral at ~pH 7.5, whereas an increased pH ~8.2 was maintained at any other time. Littoral sites had a tendency to reach a slightly higher pH 8.5–8.7 in spring to summer, prior to dropping in the fall and holding a value similar to that offshore. Of the 43 elements estimated down to ppb in limnetic regions, most were invariably low (Table 2.2). Considering metals and metal (oids), only Al and Fe were above 1 ppm, where all others were closer to ppb concentrations. Extracted photosynthesis pigments

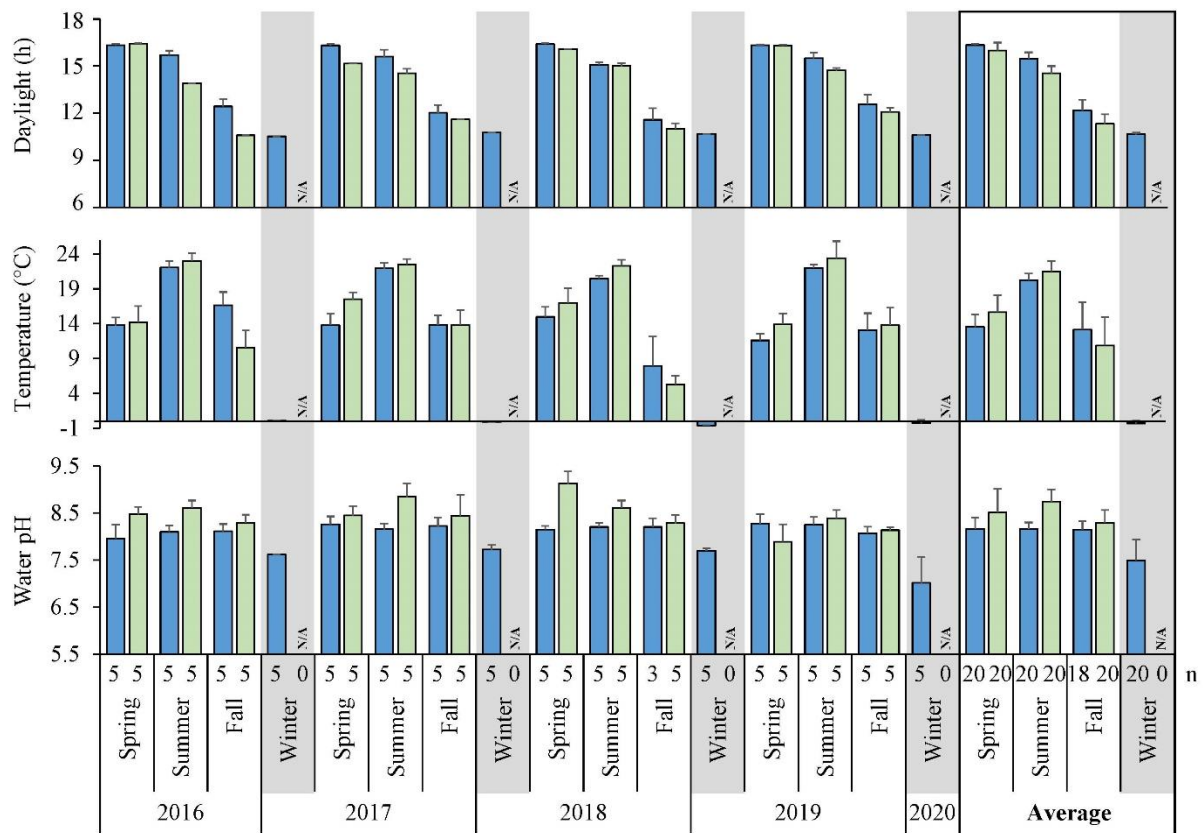


Fig. 2.2. Periodic fluctuations of lake environmental parameters. Average light availability, water temperature and pH for all limnetic (blue bars) or littoral (light green bars) samples depicted for each season. N/A, not analyzed; n, number of samples per season.

Chl *a* and *b* had highest concentrations during the summer of most years, up to 16.5 and 5.3 µg/L on average, respectively (Fig. 2.3). In comparison, levels of BChl *a* were lower and reached an average peak of 0.2 µg/L during the spring and decreased seasonally.

2.5.2. Primary productivity rates

Total carbon fixation observed *in situ* based on ¹⁴C measurements at littoral sites between 2017 and 2019 had a repeatable yearly tendency for highest rates during the spring averaging 2.00 g C/m²/day, diminishing throughout the summer to become 0.92 g C/m²/day by the fall (Fig. 2.4). Limnetic zones maintained a different pattern, where averaged offshore rates were at a minimum in the spring (0.28 g C/m²/day), gradually increasing throughout the summer to reach a fall maximum of merely 0.75 g C/m²/day. Independently, oxygenic phototrophs had a notable peak in fixation during the summer regardless of location (0.48–0.84 g C/m²/day), with reduced rates in both spring (0.15–0.57) and fall (0.28–0.40). When considering zones, shorelines maintained higher rates than offshore. Anoxygenic photosynthesis derived carbon fixation was exceptionally low across all limnetic areas (0.05–0.17 g C/m²/day), and was only marginally higher in the littoral regions (0.11–0.23 g C/m²/day). Limnetic dark reactions (chemosynthesis) increased slightly from spring through fall, 0.08 to 0.30 g C/m²/day, respectively, whereas littoral zones had the highest rates in the spring (1.21 g C/m²/day) that would later decrease, with some annual inconsistency (Fig. 2.4).

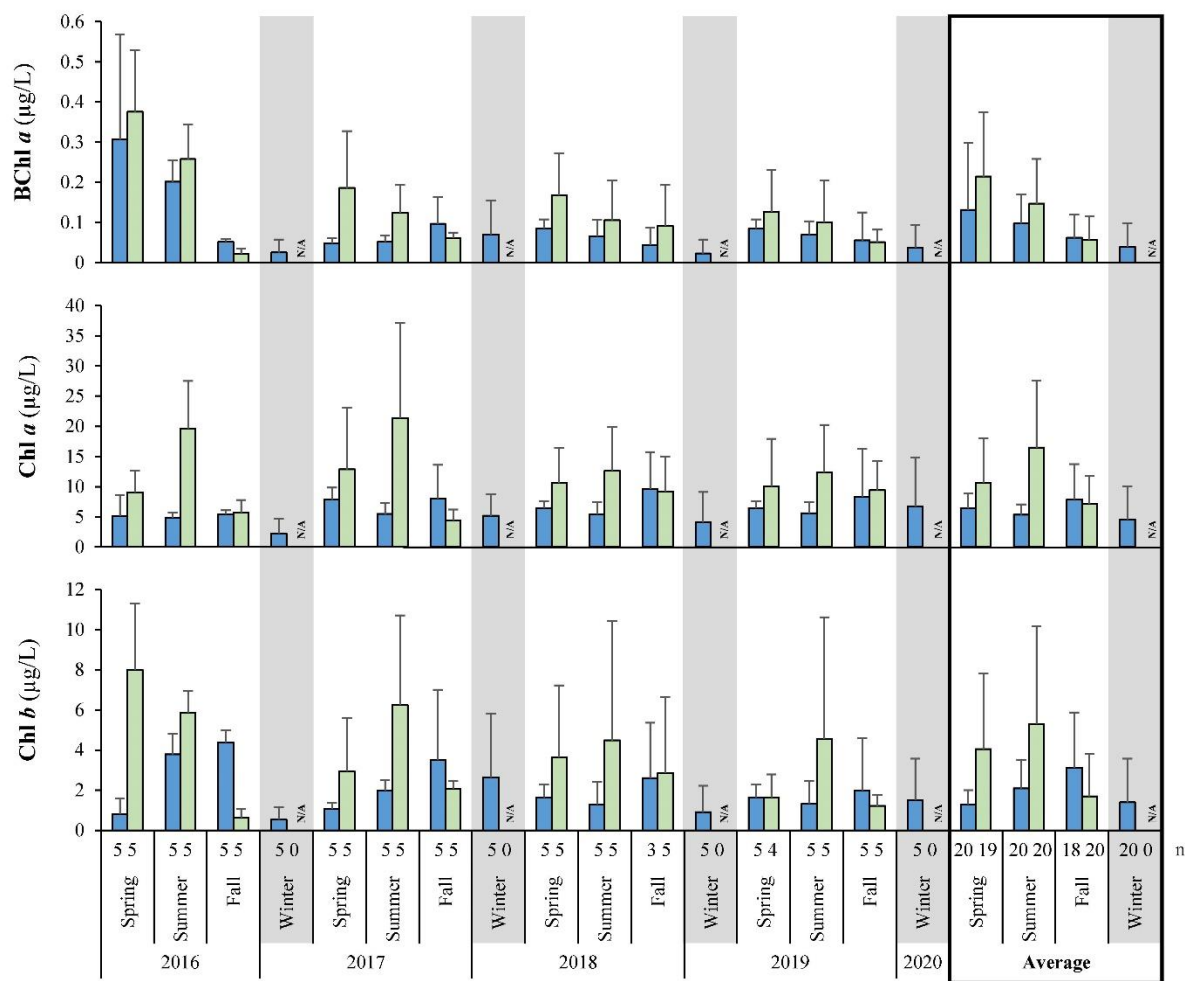


Fig. 2.3. Photosynthesis primary pigment levels from each sampling location. Solvent extracts of collected biomass revealed BChl *a*, Chl *a* and Chl *b* concentrations at limnetic (blue) or littoral (light green) zones. N/A, not analyzed; n, number of samples per season.

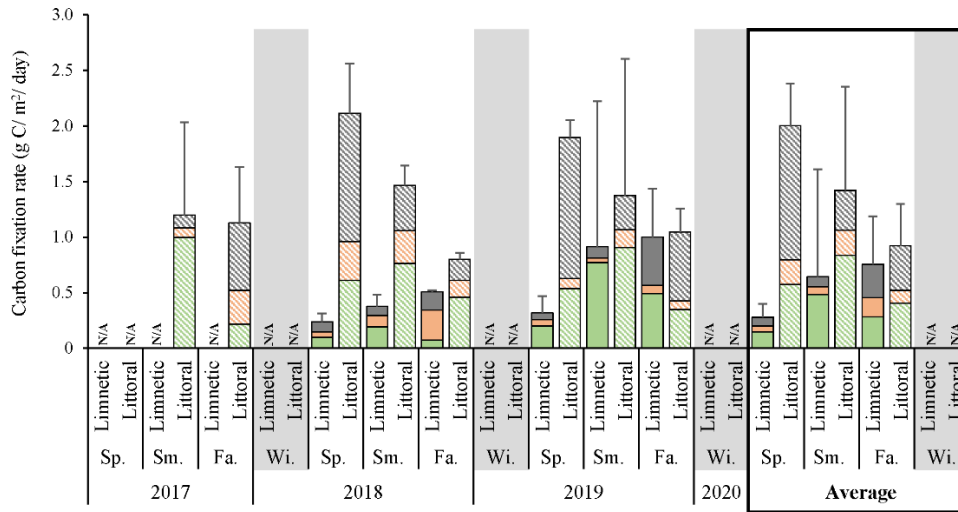


Fig. 2.4. Primary productivity of Lake Winnipeg. ^{14}C fixation measured in situ with total rates composed of oxygenic (green) or anoxygenic (orange) photosynthesis related fixation, and from dark chemosynthetic reactions (grey bars). N/A, not analyzed; Sp., Spring; Sm., Summer; Fa., Fall; Wi., Winter.

2.5.3. Seasonal fluctuations in cultivated microbial counts

Total cultured bacteria on varied media were listed (Table 2.4), with patterns in RO grown copiotrophs and oligotrophs on OM evident after repetitive enumeration (Fig. 2.5). Specifically, when grouped by zone and sample type for each season, littoral cultured bacteria peaked during the summer, while limnetic counts decreased at the same period (Fig. 2.5A). Overall, littoral sediments maintained the highest numbers on OM (4.23×10^6 CFU/g), shoreline waters had $100\times$ less (7.69×10^4 CFU/mL), and offshore counts were a further $10\times$ decreased (4.34×10^3 CFU/mL). As a general trend, more bacteria were culturable on OM than RO, regardless of season or site. Furthermore, PM and R2A media used as alternative complex organic sources to cultivate heterotrophs also maintained lesser counts than OM (Table 2.4).

AOP grown on BG-11 had a general apex during the summer of most years, to a maximum average of 9.37×10^3 CFU/g and 4.13×10^3 CFU/mL in littoral sediment and water, respectively, and would typically only reach a peak of 1.08×10^2 CFU/mL offshore (Table 2.4). Anaerobes were often below detection limits and AnAnP hovered around 5.57×10^1 CFU/mL when present. In comparison, AAP isolated and counted from both RO and OM displayed trends independent of summer maximal heterotroph numbers (Fig. 2.5B, C). The most AAP were detected in the spring of each year on either media, diminishing into the fall. The proportion of the pigmented bacteria in littoral locations reached a height of 32.9% on RO, at which time AAP also made up to 4.7, 19.1, and 11.2% of the entire cultured community of limnetic waters, littoral liquid, and sediments, respectively (Fig. 2.5B). In general, these photoheterotrophs were cultivated in highest numbers on OM in comparison to $10\times$ nutrient enhanced RO or even higher carbon containing PM (Fig. 2.6). They reached a peak of 2.39×10^4 CFU/g in the spring littoral sediments. However, AAP made up a larger proportion of the heterotrophic numbers on RO, and PM, than they did on OM regardless of sampling site (Fig. 2.5B,C, Table 2.3). Furthermore, RO_T was the most selective medium as 47.5% of cultured bacteria in sediments challenged with 100 µg/mL tellurite were AAP.

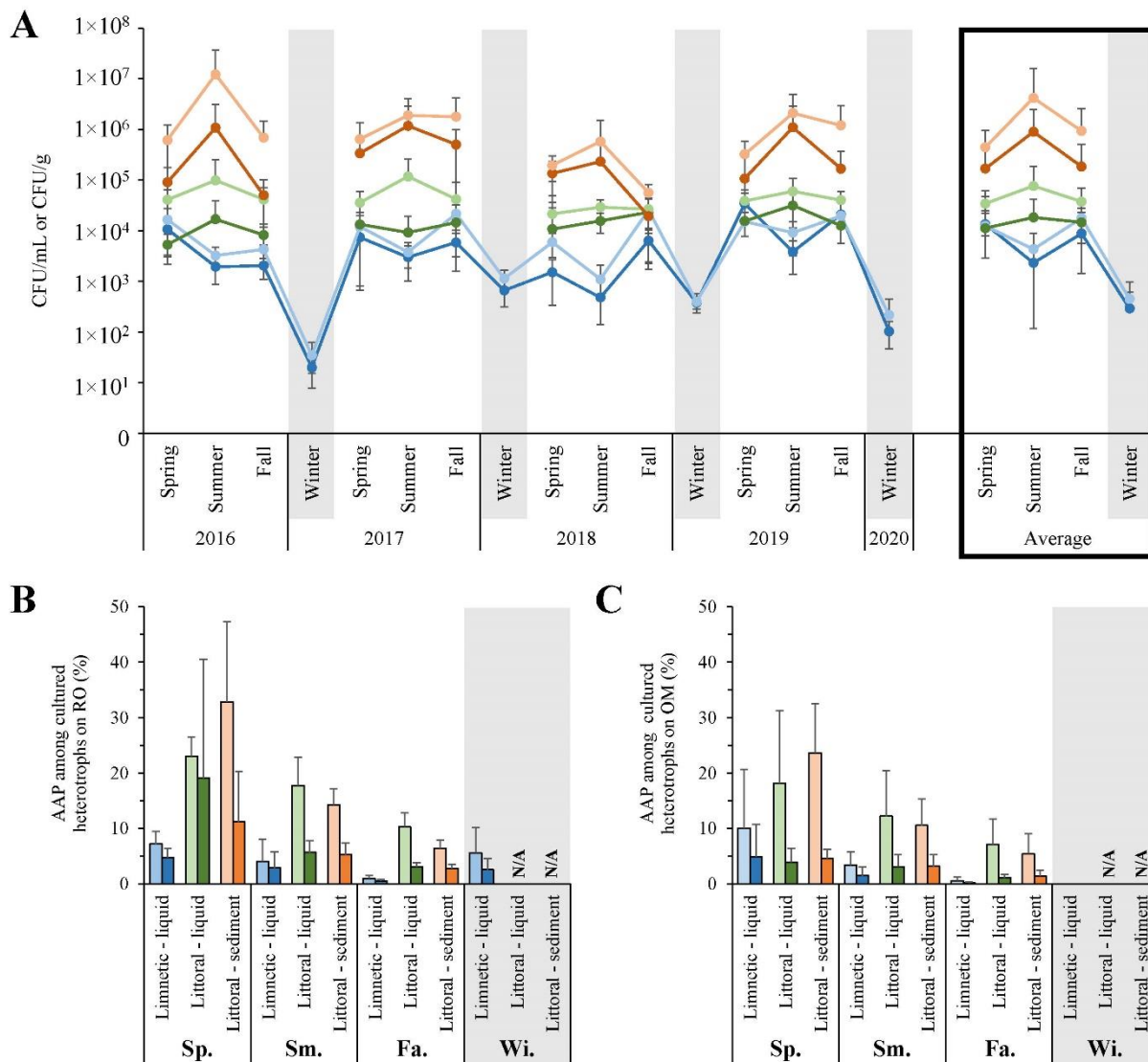


Fig. 2.5. Enumeration of heterotrophs and AAP through all seasons and years. (A) Counts of total cultured heterotrophs from limnetic water (blue), littoral water (green) or sediment (orange) samples on copiotrophic RO (dark colours) or oligotrophic OM (lighter shade of each). **(B)** Copiotrophic and **(C)** Oligotrophic AAP proportions of pigmented colonies (light shade) and total counts (dark shade) of seasonal averages from 2016 - 2020. N/A, not analyzed; Sp., Spring; Sm., Summer; Fa., Fall; Wi., Winter.

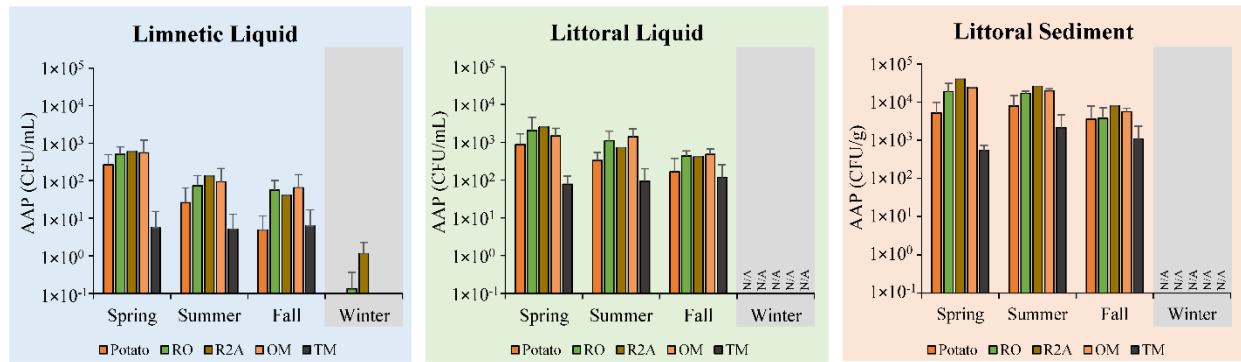


Fig. 2.6. AAP counts on various complex media. Enumerated values from all limnetic water and littoral water/sediment samples, averaged by season. N/A, not analyzed.

2.5.4. Sequenced microbial communities

Amplified DNA resulted in an average of 261,105 reads from each site. After trimming and binning, 21,877 features representing unique phylotypes were identified throughout, later distinguished via Silva132 database to 2266 taxa of species level or higher. All samples were similarly rarified when grouped by season (Fig. 2.7A), where the year 2016 reached a plateau of the highest diversity followed by 2017, then 2018–2020 (Fig. 2.7B). The dominant phyla detected were averaged by zone for each collection period (Fig. 2.8A). Considering a generalized community structure of Lake Winnipeg, the mean phyla across all sites and seasons listed from major to minor were $40.9 \pm 10.4\%$ *Actinobacteriota*, $17.5 \pm 6.6\%$ *Proteobacteria*, $9.9 \pm 6.1\%$ *Bacteroidota*, $9.8 \pm 10.2\%$ *Cyanobacteria*, $5.7 \pm 4.0\%$ *Verrucomicrobiota*, $5.7 \pm 3.6\%$ *Chloroflexi*, $4.9 \pm 3.9\%$ *Planctomycetota*, $3.4 \pm 3.1\%$ *Acidobacteriota*, $0.8 \pm 0.6\%$ *Gemmatimonadota*, $0.7 \pm 1.6\%$ *Armatimonadota*, $0.3 \pm 0.7\%$ *Patescibacteria*, $0.2 \pm 0.5\%$ *Nitrospirota*, and $0.3 \pm 0.5\%$ minimal clades. Standard deviation revealed some phyla maintained stability in the lake, while others varied dependent on location and time. Community richness and collation were exemplified using a PCoA of differing Jaccard beta diversity (Fig. 2.8C,D), where samples were found to group by season (Fig. 2.8C). A shift in composition was also detected from 2016 and 2017 to 2018 through 2020 (Fig. 2.8D). The limnetic and littoral bacteria were also differentiated as separate cohorts when visualized in PCoA (Fig. 2.9). Offshore samples were found at the extremities of the plot in tight groups, suggesting more stable communities. In comparison, shoreline representatives were both more centralized and dispersed, inferring a broad range of microbes was present with higher degrees of fluctuation.

Considering alpha diversity, monthly changes later exposed an annual rhythmic pattern (Fig. 2.8B). Overall, the highest number of different phylotypes was found during spring regardless of site. There was also the smallest variation in diversity at that time, suggesting all locations to have similarly complex microbial communities early in the year after ice break-up. By summer, bacterial heterogeneity decreased across the lake where littoral regions established the largest range in species present. While the number of individual taxa then increased at shorelines during the fall, the communities offshore steadily became more homogenous from spring to winter. Sediment bacteria had similar trends to those inhabiting the upper liquid layers at littoral zones (Fig. 2.10), which could be explained by the proximity of sampling and the frequent mixing of sand and water. However, sediment communities were distinct due to the decrease in average diversity from spring to fall that increased in variation as winter drew near, suggesting a range in community structure formed in sediments as the year progressed (Fig. 2.8B).

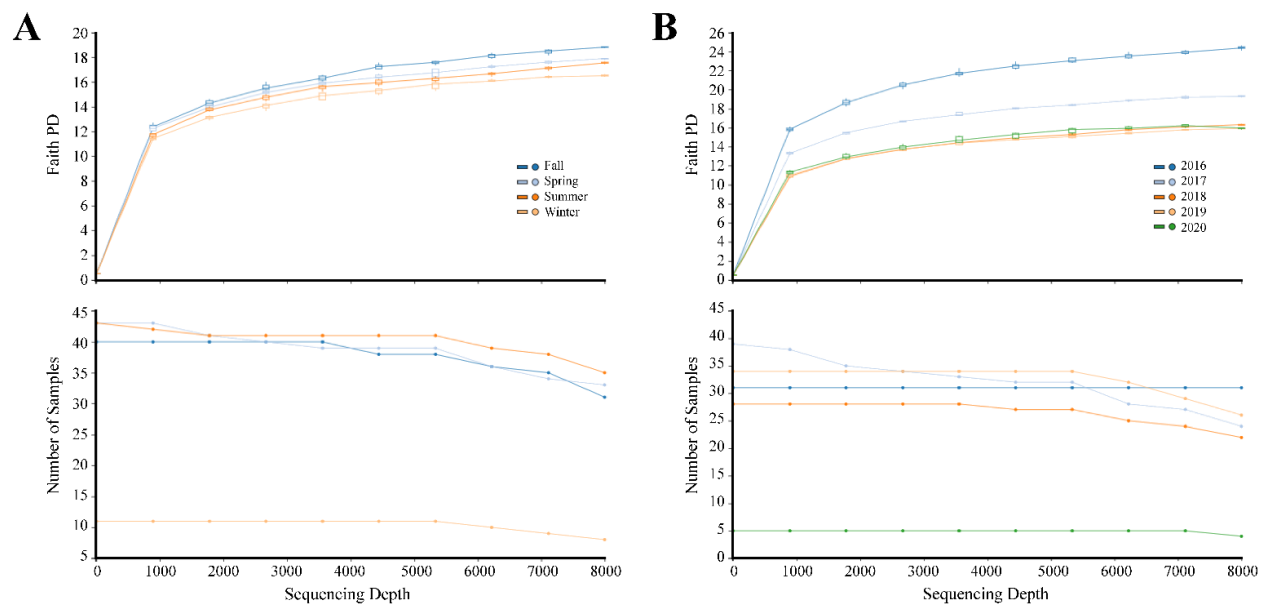


Fig. 2.7. Rarefaction curves of sequenced microbial communities. (A) Yearly and **(B)** seasonal totals of 16S V4 rRNA genes reached a plateau in diversity, when all samples reached sufficient sequencing depth.

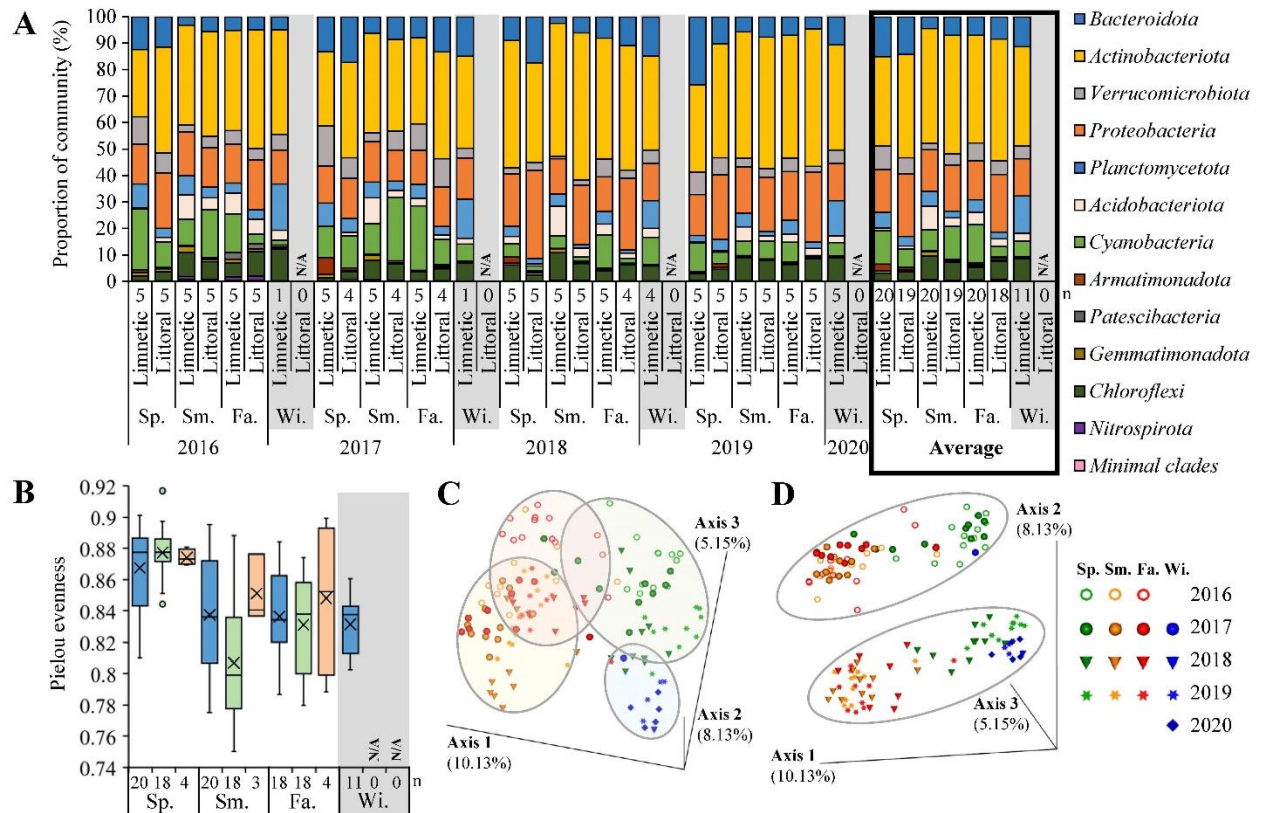


Fig. 2.8. Bacterial community composition based on sequenced 16S V4 rRNA genes. (A) Phyla in water samples defined at limnetic vs. littoral sites. Minimal clades represented less than 1% per community with *Firmicutes*, *Deinococcota*, *Bdellovibrionota*, *Crenarchaeota*, *Desulfobacterota*, *Hydrogenedentes*, *Margulisbacteria*, *Myxococcota*, *Sumerlaeota*, SAR324, MBNT15, and NB1-j clades. (B) Total α -diversity of bacteria from limnetic water (blue), littoral water (green) or sediments (orange). (C and D) PCoA ordination based on Jaccard diversity distance matrix with percent variation as principal coordinates split on indicated axis. (C) From above axis 1 and 3 separate seasons, while (D) Depicts major shift occurring along axis 2 by year with ellipses as visual cues. N/A, not analyzed; n, number of samples per season; Sp., Spring; Sm., Summer; Fa., Fall; Wi., Winter.

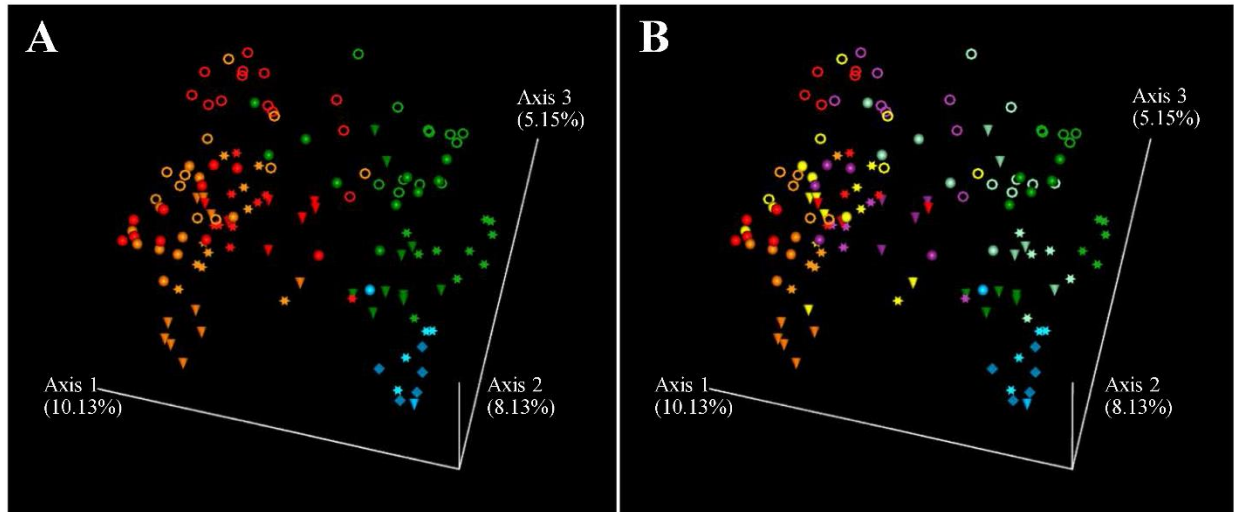


Fig. 2.9. Alternative colouration of PCoA ordination based on Jaccard diversity distance matrix. (A) Seasons included: Winter, blue; Spring, green; Summer, orange; Fall, red. **(B)** Euphotic zones separated: Winter limnetic, blue; Spring limnetic, green; Spring littoral, teal; Summer limnetic, orange; Summer littoral, yellow; Fall limnetic, red; Fall littoral, purple.

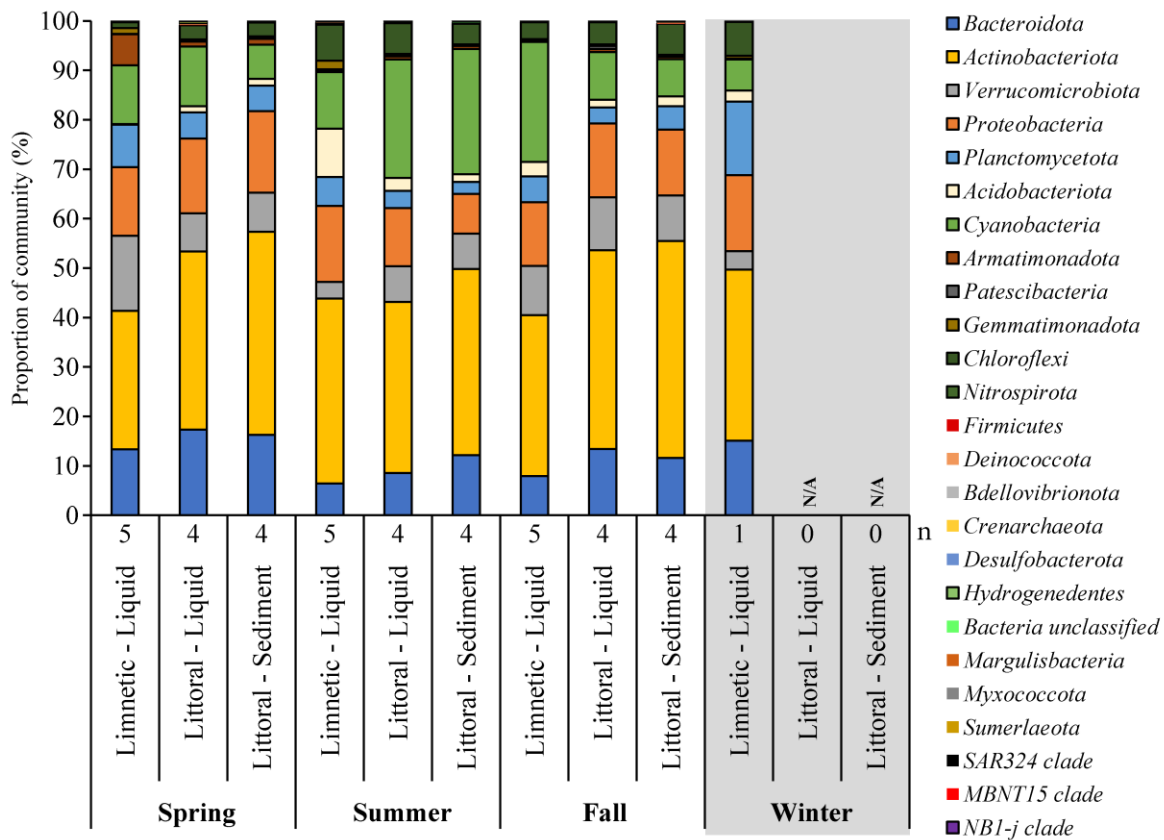


Fig. 2.10. Littoral community composition of sediment compared to liquid samples. Zone averages from Spring 2017 to Winter of 2018. N/A, not analyzed; n, number of samples per season. *, Minimal Clades (<1%).

Based on 16S V4 rRNA gene sequences, the populations of AAP, AOP, and AnAnP were individually grouped to identify and assess their changing proportion in the bacterial community (Fig. 2.11). An updated list of known AAP or AnAnP was used, Tables 1.1 and 1.2, respectively, and sequences relating 99% or higher to species or genera of either group were binned and compared to the total community. Seasonal patterns were found for each group, where AAP averaged 1.02 and 1.51% of the limnetic and littoral sequenced communities in the spring, respectively, dropping to 0.83 and 0.90% during the summer, and further to 0.41 and 0.54% by fall (Fig. 2.11). AOP that included algae and cyanobacteria, made up approximately 10% of the sequences from limnetic sites at any given time, dipping during summer and winter, while inversely increasing at littoral zones midsummer (Fig. 2.11). AOP numbers fluctuated substantially between sites and seasons, as depicted by large variations in standard deviation, whereas AAP were present and distributed more stably. The spring of 2018 was an exception, when records for *Blastomonas* related AAP were exceptionally high. AnAnP were quite nominal in comparison to other phototrophs during open water months, and only saw an increase in winter, dissimilar to the other populations (Fig. 2.11).

While all specific clades and their relative proportions were provided in Supplemental Material, the three most prevalent AAP-associated genera in sequenced communities were *Gemmatimonas*, *Roseomonas*, then *Blastomonas*. For AOP, *Cryptomonadaceae*, *Aphanizomenon*, and *Aulacoseira* were predominant, whereas the highest AnAnP were *Rhodoferrax*, *Roseiflexus*, and *Rhodoplanes*. Among known AAP (Table 1.2), *Rhodobacteraceae*, *Comamonadaceae*, and *Acidobacteriaceae* were not detected. AnAnP were exceedingly rare, with no examples of *Heliobacteria*, *Chlorobiaceae*, *Ectothiorhodospiraceae*, *Chromatiaceae*, or *Halorhodospiraceae* found.

2.6. Discussion

2.6.1. Habitat features

Lake Winnipeg has been regarded as atypical due to its shape and dimensions as the 11th largest freshwater reservoir in surface area, yet 25th by volume. Shallow in comparison to other great lakes, it has maintained a well-mixed state, where oxygen often penetrates to the bottom. As a result, thermocline-based stratification has been infrequent in the South Basin (Wassenaar, 2012). Indeed, during our study oxygen was well dispersed during open-water seasons (Table 2.2). As Lake Winnipeg is north of the 49th parallel, the duration of daylight was dependent on the time of year (Fig. 2.2). Water temperature lagged behind the spring peak of available sunlight, instead reaching height in the summer, likely due to the thermostability of the vast volume of water as a slow conductor of heat. The most significant pH change was observed at

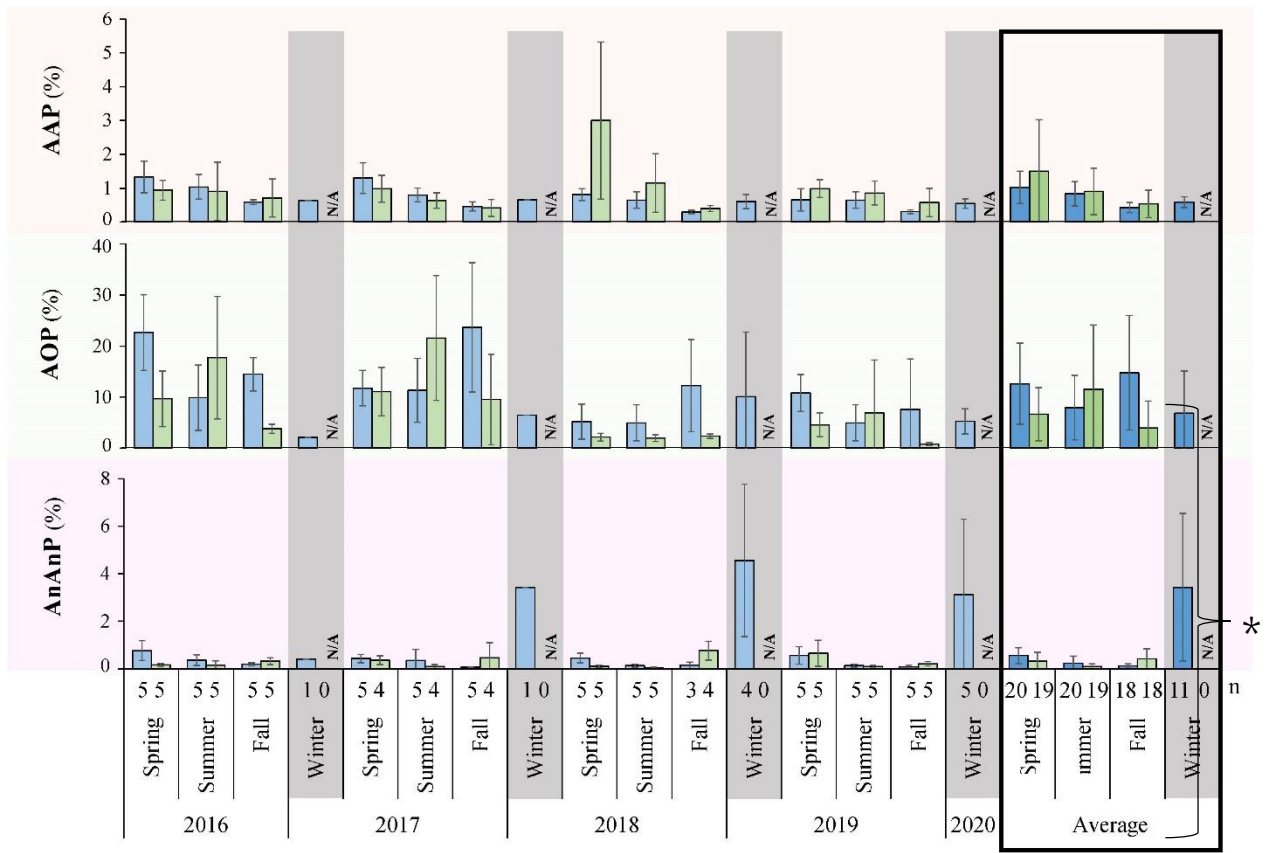


Fig. 2.11. Relative proportions of known phototrophs in sequenced communities. All 16S V₄ rRNA genes of species and genera representing AAP, AOP, or AnAnP at limnetic and littoral sites summed for each season. N/A, not analyzed; n, number of samples per season.

limnetic zones, where a drop to 7.5 occurred in winter. That decrease, and the slight additional alkalinity of pH 8.3–8.7 found in littoral areas over offshore values around 8.2 may have been due to respective water temperatures. Shallow waters had less exchange, and the warmer conditions likely increased solubility of carbonates and/or promoted activity of microbes, such as photosynthesis and biological carbon fixation, decreasing pH. Regardless, the yearly changes of these parameters (Fig. 2.2), matched previously reported years (MWSD, 2011; MARD, 2020). Furthermore, most metal concentrations were typically below 1 ppm and detection limits, confirming no significant toxic influence on life (Table 2.2). Chl *a* and *b* curves indicating AOP followed that of temperature rather than daylight (Fig. 2.3), and the averaged Chl *a* peak of 16.5 µg/L was similar to summer maxima observed via satellite imagery from 2002–2011 (Binding *et al.*, 2018). While BChl *a* from anoxygenic phototrophs was nearly undetectable and often below 1 µg/L, a common occurrence in oxic lakes (Mašín *et al.*, 2008), its low levels were still recognizable and matched daylight more so than temperature or the levels of other chlorophylls.

2.6.2. Primary productivity in Lake Winnipeg

The seasonal *in situ* carbon fixation analysis of whole community productivity found littoral microbes fixed at higher rates than those taken from limnetic zones (Fig. 2.4). These increased values were likely due to the warmer nearshore waters during the spring and summer, as temperature has been shown to affect oxygenic phototrophs (Robarts and Zohary, 1987; Sayers *et al.*, 2020), and thus may affect all carbon fixation similarly. Moreover, the maximal littoral rates decreased each fall, correlating to light availability, confirming its additional impact to fixation levels. Independently, AOP fixation was predominant during summer months, tracing the measured Chl *a* and *b* concentrations in addition to general temperature trends. In comparison, AnAnP associated productivity were marginal regardless of season, likely inhibited by the oxic nature of the habitat. Furthermore, AnAnP related rates did not correlate with BChl *a* data, suggesting this pigment was unlinked to primary productivity.

The total fixation rates at shorelines reached a spring average high of 2.0 ± 0.1 g C/m²/day (Fig. 2.4), matching recently reported values for Lake Winnipeg calculated via oxygen production rather than using carbon isotopes (Yezhova *et al.*, 2021). When compared to satellite imagery-based findings from the other 11 largest freshwater lakes, Lake Winnipeg rates determined here were greater than all, yet were most equivalent to Lake Erie's estimated mean fixation of 1.2 g C/m²/day (Sayers *et al.*, 2020). Due to the well-known eutrophication of Lake Winnipeg, and confirmed Chl *a* levels that matched recent measurements, it

may indeed currently have the highest rates among all great lakes. However, the relatively elevated numbers in Lake Winnipeg may simply be a result of underestimated values calculated in the recent satellite survey of other lakes, which was based solely on Chl *a* estimate without considering other types of carbon fixation. Indeed, a better match was found to earlier isotope-measured records of the Laurentian Great Lakes (Vollenweider *et al.*, 1974). Specifically, Lake Ontario monthly dynamics aligned well with our three time points taken yearly, where littoral regions had maximal fixation rates in the spring, which occurred prior to the swell apparent at limnetic zones. Inversely, limnetic areas were slower to warm and cool, likely the cause of delayed fixation rate peaks. However, since Lake Ontario has also suffered from nutrient loading in its recent past, and primary production may have fluctuated, future work should include a combination of satellite imagery on Lake Winnipeg in addition to chemical isotope analyses to better support our conclusions and comparisons to other great lakes.

2.6.3. Trends in cultivated microbial numbers

The height of heterotrophic cellular counts matched the peak of temperature during summer at all nearshore sites (Fig. 2.5A). Littoral sands had 4.23×10^6 and 9.05×10^5 CFU/g, while its waters maintained 7.69×10^4 and 1.84×10^4 CFU/mL on oligotrophic and copiotrophic complex media, respectively, which matched previously reported values in freshwaters and sediments (Frischer *et al.*, 2000). In comparison, limnetic numbers did not coordinate with temperature. This incongruence may have been due to the relative low of organic production rates observed offshore, when compared to nearshore (Fig. 2.4). Since limnetic regions were slow to warm, autotrophic production of excess organic carbon fixation was delayed, which in turn pushed back the bloom of heterotrophs till the fall. When considering concentrations of supplied organics, higher cell counts were found on oligotrophic OM rather than rich RO from all locations and times (Fig. 2.5A), indicating that the natural lake bacteria were adapted to lower nutrient availability. In addition, cultivated numbers on PM were 10–100× lower than RO suggesting even fewer organisms preferred the highest level of organics provided (Table 2.4). R2A enumerated values fell between those counted on OM and RO, which also coincided with its organic content in between both custom-made media. Taken together, OM clearly provided the most ideal growth conditions by incurring the highest numbers of heterotrophs, that in-turn best represented the microbial community metabolism. Of note, carbon was observed at ~0.025 g/L in lake waters (Table 2.2), and OM had 0.1 g/L, about five times higher. This would suggest, that while common laboratory practices promote bacteria growth by supplying high levels of nutrients, more dilute concentrations closer to environmental conditions may be ideal for the cultivation of highest numbers of

heterotrophs. While the amount of carbon in Lake Winnipeg were considered eutrophic (MARD, 2022), these findings also suggested that the current microbial community is accustomed to oligotrophy, and do not have preference to even higher levels of nutrients. Future work should include more dilute media for enumerations, as such composition may outperform the conditions provided in standard RO or previously considered oligotrophic OM. Follow-up microscopy may also shed light on the microbial content since cultivated counts fell short of microscopic observations from other Canadian lakes by 10–100× (Fauteux *et al.*, 2015). Later investigation could examine alive/dead cells, or if the additional microbial content had specific growth requirements not provided in laboratory.

Regarding phototrophs, few AnAnP were detected along with strict anaerobes (Table 2.4), presumably a result of the high inhibitory oxygen content. AOP were cultivated up to 9.37×10^3 CFU/mL on average, peaking in summer littoral sediments and traced both the Chl *a* and *b* levels as well as the AOP associated fixation rates. The curiosity about metals in the lake, particularly metalloids, led to the study of tellurite tolerance by the aerobic heterotrophic community. Since the toxic metalloid oxide tellurite was not present in significant amounts (Table 2.2), it was surprising to find ~5% of all heterotrophs at any given site or season capable of tolerating at least 100 µg/mL, a substantially high level (Table 2.4). While it had been previously postulated that 1 µg/mL of tellurite was toxic to most tested bacteria (Yurkov *et al.*, 1996), our findings suggested tolerance to higher levels may be more common than previously considered. Furthermore, since 47.5% of heterotrophs cultivated on RO_T were found to be AAP, this suggested that the medium was selective for some phototrophs, and that they may have metal transformation capabilities as part of their niche.

Compared to other photosynthesizing populations, cultured AAP counts neither followed trends in temperature dynamics and total heterotrophs, nor did they match measurements of AOP. Instead, they reached a pinnacle during the spring as 19.1 and 4.7% of total copiotrophs (Fig. 2.5B), or 3.9 and 4.9% of oligotrophs (Fig. 2.5C), at littoral and limnetic locations, respectively. The proportion decreased till winter each year and directly coincided with the hours of available sunlight, BChl *a* concentration, and shoreline rates of total carbon fixation. Such correlations were reasonable to find for AAP as these bacteria presumably required sunlight, and their inability to fix CO₂ or act autotrophically meant they had to rely on other community members to provide organics (Yurkov and Beatty, 1998). In addition, AAP numbers did not match up with AnAnP associated fixation rates, dark (chemolithotrophic) reactions, or AOP fixation rates

independently. Rather, AAP numbers were highest at shorelines, and particularly coincided to the maximal rates of total fixed carbon productivity (Fig. 2.4). AAP may thus be coordinated with organisms capable of fixing inorganic carbon, by consuming excess nutrients released by autotrophic neighbors in euphotic zones. Moreover, while more AAP were culturable on organic limiting OM, it was evident that they made up a larger proportion of bacteria capable of growth on the richer RO or PM (Fig. 2.5 and 2.6). Obviously, they had a tolerance/ability to grow with excess nutrients, whereas several other heterotrophs did not. Hence, aerobic phototrophs were influenced by the environmental factor sunlight, and had advantage when organics were plentiful.

2.6.4. Sequenced community analysis

General Community Composition: Culture-independent analysis of 16S rRNA genes extracted and sequenced from each location indicated a rather stable bacterial community existed across the lake (Fig. 2.8), which was reasonable as the habitat has been known to be well-mixed, therefore similarities between samples were expected (Wassenaar, 2012). While Lake Winnipeg contained a bacterial composition comparable to the average freshwater basin where *Actinobacteria* were dominant and β -*Proteobacteria* made up a large proportion (Newton *et al.*, 2011), it was very similar to a particular few great lakes of North America. Lake Erie has been found analogous both in equivalent physical surface area and volume. In addition, it contained a similar distribution of bacterial phyla at limnetic sites during the summer (Mou *et al.*, 2013). However, there were vastly different cyanobacteria described. While only 0.2–0.8% of Lake Erie's microbial community belong to AOP, and predominantly contained *Planktothrix*, Lake Winnipeg mostly had *Cryptomonadaceae*, and 7.9–11.6% of its spring to summer community were AOP (Fig. 2.11). This divergence may have been due to the irregular presence of AOP in the ecosystem, as blooms were well known to unevenly disperse yearly and spatially because of several factors including wind currents (Kling *et al.*, 2011). In Lake Winnipeg, a large variance in AOP indicators was found. Chl *a* and *b* concentrations (Fig. 2.3), AOP cultivated numbers (Table 2.4), and proportions of sequenced communities (Fig. 2.11) each had dispersed standard deviation, which could be explained by their tendency to move in clumps and not be homogenous in every liter of lake water. With such considerations, the Lake Erie analysis may have missed these populations by only sampling once at limited locations. Indeed, a more recent comprehensive study quantified cyanobacterial blooms in Lake Erie using specific 16S rRNA primers for AOP when sampled in the fall (Jankowiak *et al.*, 2019). These findings were both coherent with ours and more comparable to other

lakes (Yan *et al.*, 2017), suggesting future work dedicated to AOP in Lake Winnipeg may be required to assess pelagic apart from aggregate forming populations.

Community Fluctuations: Repeating cyclical patterns were evident for Lake Winnipeg's bacterial populations (Fig. 2.8C). Winter and spring samples individually had distinct groups, whereas summer and fall overlapped considerably. The warmer months likely developed homogenous compositions, as similar seasonal dynamics reaching a climax community has been observed in other locations with relatively comparable freeze-thaw cycles (Yan *et al.*, 2017). Lake Winnipeg's distinct winter structure was likely influenced by the drastic habitat transformation, that had low temperatures and an encapsulating frozen surface for several months (Fig. 2.1B). While seasons returned to similar repeated conditions present in previous years (Fig. 2.8C), a total diversity migration was observed from 2016 to 2020 (Fig. 2.5D). Results for both 2016 and 2017 were similar, whereas 2018–2020 clustered apart. A noticeable transition occurred in 2017, as its rarified diversity was between the 2016 maximum and 2018–2020 minimum (Fig. 2.7B). Since most monitored environmental factors remained consistent across all 5 years (Fig. 2.2), it was doubtful they caused the drift in bacterial composition. Instead, such a shift may have been due to the recent infestation by zebra mussel bivalves, *Dreissena polymorpha*. These invasive species were first spotted in 2013, but then undoubtedly confirmed in 2015. Perhaps their filter-feeding activity and rapid consumption of bacteria caused the slow change of microbial community composition (Depew *et al.*, 2021; Frischer *et al.*, 2000), and decreased microbial diversity via selective grazing. Further monitoring of bacterial communities is therefore encouraged to see if this shift continues, or if natural decade-long fluctuations have been prone to occur in the habitat.

Coordination of Phototroph and Environmental Dynamics: Littoral AOP were observed maximally in the summer making up a significant proportion, 11.5% of the sequenced community (Fig. 2.11). This followed the detected Chl *a* and *b*, highest AOP associated carbon fixation rates, as well as trends in water temperatures. They did not match the length of available daylight in spring, instead being delayed till summer. As suggested previously, temperature dependent growth and autotrophy contributed to this delay, rather than following available sunlight patterns (Robarts and Zohary, 1987). As limnetic zones were even slower to warm than nearshore (Fig. 2.2), lower proportions of AOP and associated fixation rates midsummer were expected and observed (Fig. 2.4, 2.11). As for AnAnP, they made up a minimal part of any sequenced community during open water seasons (<0.5%), but peaked in winter at limnetic sites (Fig. 2.11).

This may have been a result of the limited number of total active organisms throughout colder months, as indicated by the few cultivated heterotrophs and other bacteria (Fig. 2.5A; Table 2.4). If all heterotrophs and AOP had a winter decrease, perhaps the persisting AnAnP represented a higher proportion. As oxygen was present in the lake, even under frozen ice conditions (Table 2.2), these results stimulate future research to understand why certain AnAnP clades made up larger winter values.

The sequenced AAP of the community maintained a seasonal pattern similar to that of the culture-based enumerations, maintaining the highest proportion in the spring, decreasing throughout the year (Fig. 2.5B, C and 2.11). Up to 19.1% of the cultured heterotrophs on RO were AAP, whereas only 1.5 – 3.0% of the total sequences were from these phototrophs in spring shoreline waters. The diminished values estimated by sequencing suggested not all species present could grow on the provided RO or OM media. However, the corresponding trends between both techniques strongly inferred that cultivation reflected a fair vision of the microbial community, and that a majority of the bacteria were likely aerobic heterotrophs. In addition, the combination of applied methods permitted several comparisons to other lake studies. The highest cultured AAP count was 2.39×10^4 CFU/mL (Fig. 2.6), a number similar to mid-summer microscopic observations of other Canadian lakes (Fauteux *et al.*, 2015). Seasonal fluctuations of AAP have been analyzed infrequently in large freshwater habitats, with notable studies from the small Čertovo and Vlkov Lakes, Czechia (Mašín *et al.*, 2008; Kolářová *et al.*, 2019), and alpine Gossenköllesee Lake, Austria (Čuperová *et al.*, 2013). In each case a culture-independent autofluorescence technique was used to detect anoxygenic phototrophs, reporting AAP to be prevalent in the warmest months. The latter two studies also correlated their high numbers to dissolved organic carbon trends. Dependency on available nutrients was further confirmed when AAP were additionally shown to follow diel changes of sunlight and carbon fixation in a natural setting (Fecskeová *et al.*, 2019). Such associations were notable, as they supported our results produced from Lake Winnipeg. Here, AAP peaked during the spring, persisting throughout the open water seasons. Their numbers were directly associated with the amount of daylight available (Fig. 2.2), which also aligned to when the most carbon was fixed at shorelines (Fig. 2.4). These comparable trends strongly suggested that AAP followed seasonal fluctuations reflecting on each part of their namesake. When there is ample oxygen, light, and organic carbon, they thrive. A recent pertinent study of several freshwater lakes in Quebec, Canada reported that while AAP numbers followed light availability, they negatively correlated to water clarity (Fauteux *et al.*, 2015). This was likely due to their requirement of organic carbon, as translucent water has been indicative of an extremely oligotrophic environment, with few microbes and highly limited available nutrients.

In the area of Lake Winnipeg, the longest daylight occurred during spring, which was the same period for the highest rate of carbon fixation. This timing was therefore optimal for light-dependent phototrophs, as well as heterotrophs consuming nutrients produced by close neighbors. AAP were uniquely persistent during the early open-water season, as the conditions provided a selective advantage for them to thrive. Of note, some growth experiments have reported AAP to promote BChl a production only after incubation at refrigerant temperatures (4-8°C) (Csotonyi *et al.*, 2008), which may suggest adaptation to the colder spring, when sunlight was present. The low presence of AAP estimated in sequenced winter communities and cultivated on plates both revealed cold temperatures played a minor inhibitory role. The analysis of additional habitats far from the equator that maintain separate daylight and temperature shifts may help to confirm which parameters are most influential to AAP growth and photosynthesis. Furthermore, the analysis of isolates from such environments and their thermal tolerances in addition to pigment synthesis may elude how influential these factors are on growth.

AAP Diversity in Lake Winnipeg: Of the known taxa that contain AAP (Table 1.2), Lake Winnipeg samples had representative DNA from *Gemmatimonadota*: *Gemmatimonas* and proteobacterial *Acetobacteraceae*: *Roseomonas*; *Methylobacteriaceae*: *Methylobacterium*, *Methylobacterium*; *Rhodospirillaceae*; *Sphingomonadaceae*: *Polymorphobacter*, *Blastomonas*; *Erythrobacteraceae*: *Porphyrobacter*; *Rhodocyclaceae*; and *Halieaceae*. Interestingly, no sequences were detected from AAP of the β -proteobacterial *Comamonadaceae* group, which included *Limnohabitans* and *Roseateles*. While *Limnohabitans* has been indicated in several lakes (Kasalický *et al.*, 2018), perhaps its preference for oligotrophy, and the eutrophic nature of Lake Winnipeg let other organisms to out compete them. Furthermore, no AAP from *Rhodobacteraceae* were found in the sequenced communities. Likely, along with *Roseateles* (Suyama *et al.*, 1999), this was because most known examples such as *Roseobacter* (Shiba, 1991), *Roseicyclus* (Rathgeber *et al.*, 2005), and *Charonimicrobium* (Csotonyi *et al.*, 2011) hailed from marine and salt water habitats. In addition, no representatives of the newly discovered saline-preferring prosthecate AAP *Photocaulis* of *Maricaulales* were detected (Kuzyk *et al.*, 2022). Since Lake Winnipeg is freshwater, the presence of marine AAP was not expected. Finally, the unusual *Acidobacteriota*: *Chloracidobacterium* were also not observed. This was predicted since the representative type species was isolated from a hot spring, being thermophilic and microaerophilic (Tank and Bryant, 2015), whereas Lake Winnipeg had quite moderate temperatures, and was well-aerated (Fig. 2.2, Table 2.2). Future analysis of the Lake Winnipeg DNA dataset may reveal additional AAP species when reference databases are updated. Later work on isolated and purified strains

from this habitat could also identify each taxon observed in the sequenced community, shedding light on their individuality and physiology of the diverse species present.

In summary, the well-mixed nature of Lake Winnipeg has maintained stable microbial communities that fluctuate seasonally with a common cyclical pattern, returning to an established composition each year. Littoral shorelines and limnetic open water had distinctly separate population dynamics, containing related yet specific diversity and culturable numbers of heterotrophs. While AOP were variably present, AAP made up a stable important component of the microbial community highest in the spring, decreasing throughout the year. Their levels clearly coincided with the available daylight in all areas, BChl *a* levels, and particularly with total carbon fixation rates at littoral zones, confirming that they were promoted when light and organic carbon is available. In comparison, temperature, pH and physico-chemistry including metal concentrations may only have minor impacts on their population dynamics. Future research will corroborate if similar factors play important roles for AAP ecology in other habitats.

2.7. Acknowledgements

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3. Chapter 3.

Association of aerobic anoxygenic phototrophs and zebra mussels, *Dreissena polymorpha*, within the littoral zone of Lake Winnipeg's South Basin

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3.0. Preamble

As we were studying AAP in Lake Winnipeg, we learned that an invasive species *D. polymorpha*, was introduced in the middle of our 4-year long lake sampling. With an unknown predation profile, there was concern as to how zebra mussels may affect the ecosystem. Literature review revealed that bivalves potentially consumed bacteria, therefore we investigated them to see if they were indeed impacting AAP or other populations among the microbial community.

3.1. Contributions statement

Conceptualization, S.B.K. and V.Y.; methodology, S.B.K. and V.Y.; software, S.B.K.; validation, S.B.K., V.Y.; formal analysis, S.B.K., K.W., and X.M.; investigation, S.B.K., K.W. and X.M.; resources, V.Y.; data curation, S.B.K.; writing—original draft preparation, S.B.K.; writing—review and editing, S.B.K., V.Y.; visualization, S.B.K. and V.Y.; supervision, V.Y.; project administration, V.Y.; funding acquisition, V.Y.

Project was conceptualized by S.B.K. and V.Y. as a part of PhD program for S.B.K. to be performed with help of a Honours Project student K.W. Thus, zebra mussel sampling occurred at the same time as littoral sampling described in Chapter 1. S.B.K., K.W. and X.M. collected samples, and enumerated bacteria. S.B.K. and K.W. analyzed physiology. Once K.W. Honours Project concluded, S.B.K. completed all DNA work, microscopy, and quantitative analysis to make final conclusions with V.Y. and wrote the article/ chapter.

3.2. Abstract

While nutrient loading has affected all levels of Lake Winnipeg's ecology, its greatest influence has likely been on the microbial community. In addition to eutrophication, zebra mussels (*Dreissena polymorpha*) have recently invaded the ecosystem and threaten food web dynamics. Their filter-feeding predation and association with bacteria, specifically phototrophs, was investigated. A sampling trip to Lake Winnipeg in October 2017, focused on the isolation, enumeration, and identification of aerobic anoxygenic phototrophs in littoral water, sediment, and tissues of mussels. Gimli, Patricia, and Grand beaches, separated by >15 km across the South Basin, had similar bacterial counts when cultivated on rich organic, BG-11, purple non-sulphur, and K₂TeO₃-supplemented media. Culture-based enumeration on rich organic medium revealed 1.74% of heterotrophs from littoral waters were aerobic anoxygenic phototrophs, and represented 13.98% within sediments. In contrast, 0.48, 1.15, and 0.16% of cultured heterotrophs were aerobic anoxygenic phototrophs within zebra mussel gill, gut, and gonadal tissues, respectively. High-throughput sequencing of bacterial 16S V4 rDNA maintained similar trends in each bivalve organ, where 0.22, 1.13, and 0.20% of total 16S genes belonged to these phototrophs. Several *Sphingomonadaceae* isolates were recovered from gut tissues, all with filamentous morphology large enough for predation. Bioaccumulation of metals was also studied in *D. polymorpha*. All tested associated aerobic anoxygenic phototrophs were capable of tolerating the metalloid oxide tellurite. The consistent distribution of aerobic anoxygenic phototrophs within microbial communities across Lake Winnipeg, and their predominance in the gut tissues of zebra mussels suggested bacterial consumption by this invasive species.

Keywords: Aerobic anoxygenic phototrophs; Microbiome; Zebra mussel; *Dreissena polymorpha*; Metalloid oxides; Lake Winnipeg

3.3. Introduction

Lake Winnipeg, located in the province of Manitoba, Canada, is the eleventh largest freshwater lake in the world by surface area (Fig. 3.1A). Nutrient loading across its vast watershed has led to a significant increase in phosphate and nitrate, causing eutrophication (Matisoff *et al.*, 2016; “State of Lake Winnipeg 2nd Edition”, 2020). The lake contains two shallow basins with 12 m mean depth, which has prevented permanent stratification and kept the waterbody predominantly oxic (Kling *et al.*, 2011). In addition to the surrounding farmland, the drainage basin has many historic (Cole, 1949) and currently active mining operations (Alexandre *et al.*, 2019; Roberts *et al.*, 2020). As their environmental impact was not a consideration in the past, many remote mines were left open, resulting in tailings enriched in toxic compounds including metal(oid) oxides (Hughes *et al.*, 2017). If the leaching toxins have entered nearby waterways, there has been a potential to negatively influence the aquatic ecosystem (Sumi & Thomsen, 2001).

Nitrogen and phosphorus loading has likely caused the proliferation of many microorganisms, that were otherwise unable to grow due to the limited bioavailability of the now plentiful resources (Yuan *et al.*, 2014). Of these opportunistic organisms, chlorophyll *a* (Chl *a*) containing oxygenic phototrophic algae and cyanobacteria, have been often monitored as the result of eutrophication. A major study of the lake from 1999 to 2016 tracked the phytoplankton biomass via Chl *a* levels, which varied between 4.1 µg/L (in 2000) to 35 µg/L (in 2006), with a mean 12.2 µg/L in open water (MARD, 2020). Between 1999 and 2007, the overall microbial biomass was annually detected via microscopic analysis to be dominated by cyanobacteria and diatoms, making up 13 to 94% (MWSD, 2011). However, these primary producers of organics have not been the only group of microorganisms to take advantage of the nutrients in excess. In many instances, aerobic anoxygenic phototrophs (AAP) were found associated with cyanobacteria as co-dominant species within the mat structures (Yurkov and van Gernerden, 1993; Yurkov and Beatty, 1998; Yurkov, 2006; Bilyj *et al.*, 2014). Interestingly, AAP have made up to 18.9% of the total aerobic heterotrophic population from samples containing cyanobacteria within the microbial community of spring and fresh waters (Bilyj *et al.*, 2014; Koblížek, 2015), often exceeding 10% in marine habitats (Yurkov and Csotonyi, 2009), and potentially up to 37% of total bacteria in lakes (Fauteux *et al.*, 2015). High proportional presence at different localities world-wide has led to increasing interest in their ecological niche and importance (Koblížek, 2015).

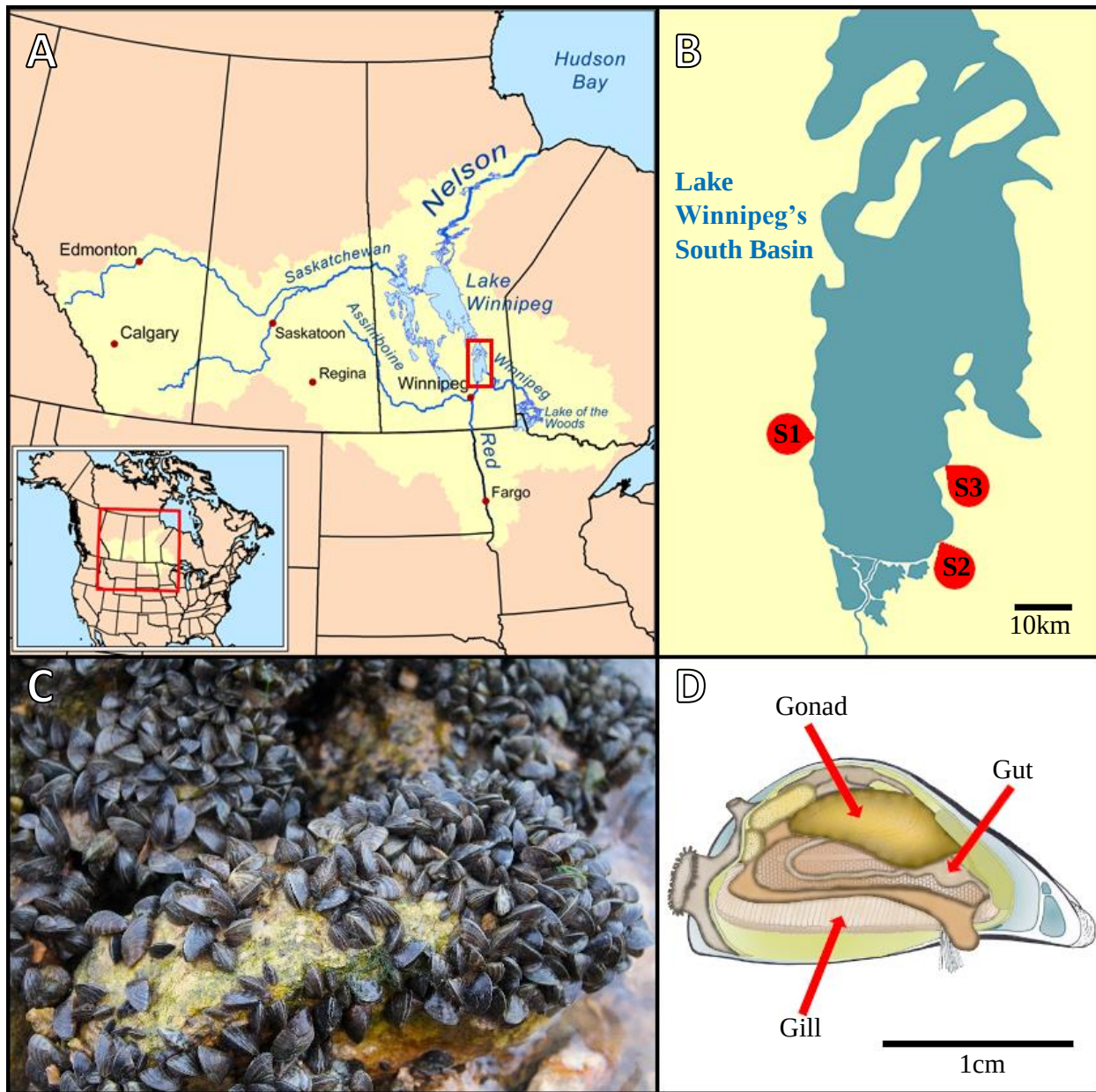


Fig. 3.1. Lake Winnipeg's drainage basin, sampling sites, and local zebra mussels. (A) Map (with kind permission from author Mr. Karl Musser, with some modifications) of the 982,900 km² drainage basin within North America, the 5th largest in the world, spans east from the Rocky Mountains through Canadian provinces Alberta, Saskatchewan, Manitoba, and Ontario, and north from the American states, Montana, North Dakota, South Dakota, and Minnesota prior to draining into the Hudson Bay. (B) Sites included Gimli Beach, S1; Patricia Beach, S2; and Grand Beach, S3. (C) Infestation of *D. polymorpha* near harbour of Gimli Beach, October 2017. (D) Schematic cross section of zebra mussel anatomy, depicting organs sampled from each bivalve.

Phylogenetically, AAP are classified within the α , β , and γ -*Proteobacteria* (Yurkov & Hughes, 2017). Although ribosomal 16S rDNA sequences are similar to purple non-sulphur bacteria (PNSB), phenotypically they are drastically different. While most known PNSB can function as heterotrophs aerobically and anaerobically, they can also perform photoautotrophy in the absence of oxygen and organic carbon. Conversely, AAP strictly require oxygen for either heterotrophic or photoheterotrophic growth. Both PNSB and AAP perform anoxygenic photosynthesis with bacteriochlorophyll *a* (BChl *a*). However, PNSB produce and use BChl *a* exclusively anaerobically, whereas AAP do so in the presence of oxygen only. In addition, AAP lack the Calvin cycle key enzyme RuBisCO and cannot fix CO₂, explaining why organic carbon must be provided externally (Yurkov and Beatty, 1998; Fauteux *et al.*, 2015). While PNSB and AAP are typically delineated, one known organism, *Charonimicrobium ambiphototrophicum*, was found to produce BChl *a* in the presence and absence of oxygen (Csotonyi *et al.*, 2011). However, due to its requirement of complex organics for suitable proliferation, the lack of complete photoautotrophy indicated its transitional status between the two groups.

Regardless, AAP are noteworthy due to their ability of supplementing heterotrophic energy production with phototrophy. This attribute can allow them to outcompete strictly heterotrophic bacteria in illuminated eutrophic environments (Yurkov and Beatty, 1998; Garcia-Chaves *et al.*, 2015; Ferrera *et al.*, 2017), or to outlast some heterotrophs in oligotrophic conditions, as they don't have the same dependence on organic carbon availability (Hauruseu and Koblížek, 2012). Interestingly, Chl *a* and BChl *a* absorb light in different parts of the visible spectrum, 600-700 and 800-900 nm respectively, which has allowed them to grow in the same habitat without much competition. Furthermore, organic carbon consumption enhanced by light energy near Chl *a* containing primary producers of organics, has indicated a carbon-recycler role for AAP within cyanobacterial mats (Michelou *et al.*, 2007).

Once a phototrophic microbial population is established in an illuminated aquatic location, their cellular biomass and released organics serve as the base of the food chain in that ecosystem (Chikaraishi *et al.*, 2009). These planktonic microorganisms are then primarily consumed by both zooplankton and filter-feeding organisms, which significantly rely on substrate size for consumption. Notably, some AAP are several times larger than many other aquatic bacteria (Fauteux *et al.*, 2015). Both their enlarged size and tendency to aggregate, potentially render them more vulnerable to grazers.

D. polymorpha is a small bivalve native to Eastern Europe and Western Russia. It has been considered as the most aggressive freshwater invader in the Northern American Hemisphere (Karatayev *et al.*, 2015), since initially infecting the Great Lakes of the St. Lawrence drainage basin in the mid 1980's. Even with precautions in place, Lake Winnipeg had its first confirmed zebra mussel spotted in 2013, with a large biomass already discovered by 2015 (DFO, 2014). These bivalves are of interest due to their filtration feeding method, where a single animal can filter up to one litre of water per day (Reeders *et al.*, 1989). In addition, adults are very small when compared to local *Mollusca*, <3 cm, and they form massive colonies upon solid substrates without a proximity limit to one another (Fig. 3.1C). An explosion in population size is therefore a concern due to the unknown consequences of increased collective feeding on the local ecosystem (MacIsaac, 1996). Although *D. polymorpha* is known to strain water for nutrition, and have a filtrate size between 5 – 45 µm (MacIsaac *et al.*, 1995; Horgan and Mills, 1997), a preference in prey remains unclear. While the tiny size of microbes (typically <3 µm) would suggest passive transport through the animal, experimental subjection of zebra mussels to *Escherichia coli* or *Pseudomonas aeruginosa* cultures revealed the animal's activity decreased bacterial titre (CFU/mL) over time (Silverman *et al.*, 1996; Mezzanotte *et al.*, 2016), with potential selection based on cellular size (Cotner *et al.*, 1995; Mosteo *et al.*, 2016). However, since the focus of these studies were on artificially added, clinically relevant bacteria, such results only permitted speculation that bivalves were consuming bacteria in nature.

In addition to assimilating nutrients from their surroundings, filter feeders have been known to accumulate toxic metal compounds (Briant *et al.*, 2016; Larios *et al.*, 2018). Furthermore, bivalves near hydrothermal vents had elevated metals in their tissues, containing symbiotic microorganisms that could tolerate, and potentially detoxify metals (Kádár *et al.*, 2006). Many AAP isolated from locations with elevated heavy metals such as hydrothermal vents (Maltman and Yurkov, 2015) and mine tailings (Hughes *et al.*, 2017) were known to survive extremely high levels of TeO_3^{2-} in the range between 500 and 2,000 µg/mL, while concentrations higher than 1 µg/mL are typically extremely toxic to most bacteria (Yurkov *et al.*, 1996). In addition, natural samples have been shown to have AAP with enlarged cellular sizes (Mašín *et al.*, 2008), that may fit within the filtration size of these mussels. The AAP presence in Lake Winnipeg, their increased cell volume enabling predatory capture, as well as an inherent ability to survive toxic metal oxides that may also be accumulated in bivalves, have all lead to investigate their association with *D. polymorpha*.

While previous works have specifically studied the enumeration of coliforms from the gut, whole homogenized mussels, or water near zebra mussel colonies (Starliper *et al.*, 1997; Mosteo *et al.*, 2016; Daeschlein *et al.*, 2015), none have simultaneously compared the bacterial community within organs to the local environmental waters and sediment. In addition, no bivalve-related study has used both classical culture-based and molecular-biological techniques in parallel. Cultivation of heterotrophic bacteria will allow us to determine the proportion of AAP among each cultured community. Analysis of individual isolates will reveal species-unique or population-wide phenotypic characteristics that may suggest a niche for this group of bacteria. Since cultivation of the entire bacterial community and all AAP members is not probable under the same conditions, a high-throughput sequencing technique of environmentally sourced DNA (eDNA) collected from each site was chosen to help elucidate the unculturable varieties of prokaryotes that are present. All in-tandem techniques can then be used to compare Lake Winnipeg's littoral zones to those reported from other eutrophic aquatic habitats (Koblížek, 2015; Kolářová *et al.*, 2019). Due to the previous detection of AAP with other grazing crustaceans, zooplankton, and protists (Bilyj *et al.*, 2014; Garcia-Chaves, *et al.*, 2015; Ferrera *et al.*, 2017), we investigated their presence within the microbiome of zebra mussels in the South Basin of Lake Winnipeg.

3.4. Materials and Methods

3.4.1. Site description and sample collection

The observation of *D. polymorpha* during 2015 in the South Basin of Lake Winnipeg led to the speculation that likely originated from the Red River. To assess microbial interactions with these bivalves, 3 sites were chosen that likely had mussel beds already established across the South Basin separated by more than 15 km to determine if similarities or differences existed near or far from the river outlet. Samples were collected on October 1st, 2017, from each location as a late season sampling would likely have adult mussels established near shoreline for collection. Site 1, Gimli Beach (50°38'1"N, 96°59'2"W) and Site 2, Patricia Beach (50°25'26"N, 96°37'2"W) are on either side of the South Basin separated by 35 km, whereas Site 3, Grand Beach (50°34'11"N 96°35'59"W) is 15 km North of Site 2 on the East side of the South Basin. All sites are primarily fed by the waters of the Red River (Fig. 3.1A and B), and therefore likely had similar nutrient availability. Temperature and pH were recorded with a calibrated Beckman ϕ 255 pH/Temp/mv meter. Water clarity was measured by Secchi disk test (Fuller *et al.*, 2004). At each site, 3 L of water and 100 g of sediment were collected in separate sterile containers allowing for head space from the shoreline, where lake water was 0.5 m deep as to acquire euphotic zone samples. All locations were chosen as primarily sandy

shorelines to maintain similar environmental analysis. Sites 1 and 2 had some visible submerged rocks along the coast, allowing for an approximately 30 cm diameter stone with numerous live respiring zebra mussels to be transferred into a sterile 5-gallon pail containing lake water. At the time of sampling, no solid substrates or attached *D. polymorpha* were encountered at Site 3, preventing the collection of mussels. Samples were then cooled on ice in the dark and transported within 4-6 h back to the laboratory for immediate processing and the following experimentation.

3.4.2. Chemical analysis of samples

To determine the major atomic composition of water, sediment, and zebra mussels, samples were subjected to collision/reaction cell based inductively coupled plasma mass spectrometry (CRC ICPMS). Water samples were treated with nitric and hydrochloric acids prior to analysis by CRC ICPMS (Martin *et al.*, 1994; U.S. EPA, 2014). Sediment was dried, disaggregated, and sieved. Strong acid leachable metals in the <2 mm fraction were solubilized by heating a sample dissolved with nitric and hydrochloric acids, to liberate naturally available metals, prior to instrumental analysis (Martin *et al.*, 1994; U.S. EPA, 2014). For the elemental content analysis in zebra mussel samples, a large quantity of tissue was required, >10 g, which prevented the screening of individual organs. Since the intent was to analyze metal bioaccumulations within the mussels including the shell, several whole organisms from each site were crushed and homogenized, and sub-sampled prior to hotblock digestion with nitric and hydrochloric acids, in combination with hydrogen peroxide, prior to CRC ICPMS (Martin *et al.*, 1994; modified from U.S. EPA, 2014). Elements and their detection limits are listed in Table 3.1.

3.4.3. Aseptic dissection of *D. polymorpha*

Once in the laboratory, three adult mussels from each site were prepared for internal microbial analysis as described with some minor modifications (APHA, 1970). In brief, our methods included individual mussels cut from solid substrate with a sterile blade and then scrubbed, cleaned, and rinsed with sterile ddH₂O to remove external bacterial film. Bivalve abductor muscle and hinge were cut, so shell could open for the removal of organs (Fig. 3.1D). Tissues were chosen to determine bacterial content in specific compartments, and to allow for the differentiation of microbes specific to the intestinal tract. As *D. polymorpha* is quite small, <3 cm, aseptic dissection was chosen to avoid compromising the inner organ microbiome. Previous work found washing tissue exterior modified bacterial flora substantially (Winters *et al.*, 2011). Hence, we chose to aseptically sample the gill, gut and gonadal tissues, avoiding any organ washing or disinfection suggested in the original method (APHA, 1970). Furthermore, gills were expected to have organisms about

to be consumed, as well as those soon to be rejected in pseudofeces. Logically, gut samples would contain both commensal and prey bacteria, whereas healthy gonadal tissues should theoretically be free of microbes. Therefore, any bacteria in the gonadal tissues would represent those of a microbiome found throughout the mantle cavity. They could then be compared to those in the gill and gut, to ascertain what bacteria likely originated from within the specific organs by negating those species from the communities observed in either other tissue. All tissue samples were weighed individually and homogenized in 10 mL of oligotrophic dilution medium using a sterile Kontes Glass Company tissue grinder.

3.4.4. Culture based isolation, enumeration and bacterial enrichments

Water, suspended sediment, and homogenized tissue samples were decimally diluted to 10^{-7} fold (Hughes *et al.*, 2017). Aliquots of 0.1 mL from each concentration were spread in triplicates on 2% agar plates. Media were designed to isolate and enumerate specific physiological groups of microorganisms, with a focus on phototrophs. Heterotrophs, including photoheterotrophic AAP, were cultivated on rich organic (RO) medium as described (Yurkov *et al.*, 1999). PNSB were enriched on a specific medium PNSM, and solid BG-11 medium was used to cultivate aerobic oxygenic phototrophs (AOP) and oligotrophic AAP as described (Bilyj *et al.*, 2014). Heterotrophs capable of tolerating toxic metalloid oxides were cultivated on RO_T medium with K₂TeO₃, a representative metalloid oxide pollutant in some environments studied previously (Maltman & Yurkov, 2015; Hughes *et al.*, 2017). RO_T included the ingredients of RO supplemented with 100 µg/mL K₂TeO₃, as this amount is considered very toxic, and only tolerant organisms would be capable of growth at such concentration. Upon autoclaving, all media were adjusted to pH 7.5, and incubated after inoculation for 7 days at 28 °C. Bacterial cultures on RO and RO_T grew in the dark, while BG-11 and PNSM plates were both kept under constant illumination. All cultures were grown aerobically, except those on PNSM, which developed in translucent BD BBL™ GasPak™ anaerobic chambers, produced by Becton, Dickinson and Co. at 7 Loveton Circle, Sparks, MD 21152 USA. The chambers used NaBH₄ and palladium pellets to catalyze the reaction to eliminate oxygen.

Pigmented and non-pigmented colony forming units (CFU) were counted as CFU/g wet weight for tissues and sediments, and CFU/mL in water (Frisher *et al.*, 2000; Mosteo *et al.*, 2016). Representatives of all coloured morphotypes were re-plated onto their original medium to verify growth and pigmentation. Due to the reduction of TeO₃²⁻ to elemental Te (Presentato *et al.*, 2019), a majority of colonies on RO_T were black, and therefore had to be streaked onto RO without tellurite to observe their natural colour. Patched pigmented

strains were suspended in 0.2 mM TRIS HCl buffer, pH 7.8 containing 70% glycerol to measure their absorption spectra between 350 and 1100 nm using a HITACHI U2010 spectrophotometer. Presence of Chl *a* and BChl *a* in cells was annotated as previously reported (Yurkov and Beatty, 1998), where strict aerobes containing BChl *a* were identified as AAP. The amount of AAP among pigmented heterotrophs was established, and this proportion was then correlated with total amounts of cultured heterotrophs on RO to determine the AAP CFU/mL or CFU/g.

3.4.5. Physiological tests

Purified isolates containing BChl *a* were tested for the capacity to grow anaerobically as described (Csotonyi *et al.*, 2011). Morphology was studied in 1 to 3 days old cultures using a Zeiss Axioscope 2 Phase-Contrast Light Microscope at 1000x magnification. Motility of cells was determined by observation of a 24 h (log phase) culture grown in liquid RO. Pigments were extracted in an organic solvent mixture, acetone:methanol (7:2), to confirm presence of BChl *a* and carotenoids *in vitro* (Yurkov *et al.*, 1994). Logarithmic or early stationary phase cultures were cryopreserved in diluted RO containing only 10% carbon source and 30% glycerol. Tolerance to K₂TeO₃ was determined by growing cells in RO with increasing amounts of oxide from 25 to 200 µg/mL. Final OD after 5 days of growth was compared for each AAP strain and the ratio of those growing in RO without K₂TeO₃ to cultures with this oxide was established. This ratio was used to record the inhibition or promotion of growth, as the concentration of oxide increased. Due to the reduction of TeO₃²⁻ to Te, with resulting blackening of bacterial culture, growth in the presence of K₂TeO₃ was measured with readings at 950 nm rather than 600 nm as described (Maltman & Yurkov, 2015). AAP isolates were subjected to other physiological and biochemical tests, including Gram staining, motility, fatty acid composition, enzymatic activity, carbon utilization, antibiotic testing, and optimal growth pH, temperature, or NaCl as described (Rathgeber *et al.*, 2005).

3.4.6. Culture-independent ribosomal 16S amplicon sequencing

Environmental DNA was extracted using a QIAGEN DNeasy® PowerWater® Kit method with minor modifications. This included filtering 0.5 L of lake water through a 0.22 µm pore-size filter and vortex shaking the filter pad with provided granite crystals for 10 min. Sediments were directly vortexed without filtration or use of granite. Homogenized zebra mussel tissues were added directly to granite crystals for a 10min vortex shake to lyse all cellular matter. In the final step, DNA was eluted in only 50 µl of buffer. The yielded DNA was subjected to ribosomal 16S V4 amplicon gene sequencing completed by MiSeq Illumina with the primer set 515F paired to 806R; 5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-

GGACTACHVGGGTWTCTAAT-3', respectively. Community structural analysis was carried out via Qiime2 computational pipeline, using Python based (McKinney, 2010) framework (Bolyen *et al.*, 2018), and BIOM format (McDonald *et al.*, 2012). Methods included importing raw data, joining paired end reads, deblurring (Amir *et al.*, 2017), and denoising (Bokulich *et al.*, 2013), prior to operational "taxonomic" unit (OTU) alignment (Price *et al.*, 2010; Rognes, *et al.*, 2016). Three clustering methods were compared: de novo, open-reference and closed-reference using the Silva132 database (Quast *et al.*, 2013). Borderline-chimeras, which are non-identical sequences that are similar to one another, were included individually in the analysis since they provided a larger diversity of speciation. Phenetic identities were aligned as feature tables (Weiss *et al.*, 2017) at all levels of bacterial genomic hierarchy, based off of bacterial taxonomy (Stackebrandt and Goodfellow, 1991; Katoh & Standley, 2013), depicted via stacked bar charts in final presentation. Diversity was statistically quantified via α - and β - diversity analysis (Faith *et al.*, 1987; Vázquez-Baeza *et al.*, 2017). Qiime2 project code text file, as well as final steps of analysis in both Qiime2 and BIOM file formats have been provided within supplemental materials.

3.4.7. Phylogeny of isolated strains

Crude DNA from each AAP isolate was extracted by phenol chloroform method (Rainey *et al.*, 1996) for full 16S rDNA gene analysis via Sanger sequencing. Primer sets included 27F and 1492R, 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3', respectively, to achieve a total length of >1400 bp per strain. Phylogenetic relatedness was determined via web-based nucleotide Basic Local Alignment Search Tool, BLAST® (Madden, 2002), to find similarity of strains with type species. For evolutionary analysis, a phylogenetic tree was constructed via MEGA X® software (Kumar *et al.*, 2018) with 10000 bootstraps (Felsenstein, 1985), using maximum likelihood to compare new isolates to a broad range of known AAP genera, based on the Jukes-Cantor model (Jukes & Cantor, 1969). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. For further clarification of their taxonomic status, a specific *Porphyrobacter* and *Erythromicrobium*-based tree was constructed using the same approach as above to identify the position of our isolates among members of the two genera.

3.5. Results

3.5.1. Study site description

At Site 1, ambient air temperature was 14.0 °C, water had pH 8.9 and temperature 9.5 °C. At Site 2, water had pH 9.0 and 13.0 °C, with ambient air temperature 17.0 °C. Site 3 had pH 8.9, while water and ambient temperatures were 13.0 and 16.0 °C, respectively. During the week leading up to the sampling trip, the average high temperature was 15.6 °C and the average low was 8.7 °C. On the day of the sampling, there was around 11 h of daylight. Sediment samples consisted of sand of varying particles, where S1, S2 and S3 had course, fine, and very fine granule sizes, respectively.

Use of CRC ICPMS detected 39 elements down to ppb for liquid samples, and ppm for sediment and tissues (Table 3.1). Within water, Ca, S, Na, Mg, and K were the only elements over 1 ppm. In comparison, most elements were over 1 ppm in sediment, with Ca as the highest, averaging around 37,113 ppm, Mg second at over 16,000 ppm, followed by Al at 882 ppm. Whole homogenized zebra mussels also had high amounts of these elements, with Ca at a striking 174,500 ppm; 1,274 ppm for Na; 630 ppm, P; 536 ppm, S; and 250 ppm, Sr.

3.5.2. Microbial culture based enumeration from sites and tissues

Culture-based bacterial counts are recorded explicitly in Table 3.3, where each CFU/ml or CFU/g represents the average of triplicate enumerations from serial dilutions. Mean environmental waters were found to have $1.82 \pm 0.40 \times 10^4$ of cultured heterotrophs, and sediments contained on average 1000× higher. Similar, but marginally higher numbers of oligotrophs were estimated at each site on BG-11 from most sites. Bacteria capable of anaerobic growth, or tolerance to tellurite were counted on average 100 or 1000× lower than found on BG-11 or RO, at $\sim 7 \times 10^1$ and $\sim 3.52 \times 10^2$, respectively.

To analyze trends of bacterial content within different zebra mussel tissues, results based on analysis of 3 animals from each site were averaged together, and graphs compared to estimates received in the vicinity of their environment (Fig. 3.2). Since microorganisms from every bivalve organ were plated in triplicate, this summed into 9 counts performed for each tissue enumeration. Calculated this way, each mussel had on average 20× more cultured bacteria within their gut, than among the gill or gonadal tissues.

Cultivated pigmented colonies were checked if they were formed by AAP (Table 3.2), which were discovered on RO medium from each sample. A higher proportions of AAP were cultivated from sediment, than from water at any given site. Gut samples also revealed a larger population of AAP, than either gill or

Table 3.1. Total concentrations of elements. Water, sediment, and whole zebra mussel homogenate collected at from Sites S1, S2, and S3.

Element:	Water				Sediment				Whole Zebra Mussel		
	Detect	S1	S2	S3	Detect	S1	S2	S3	Detect	S1	S2
Aluminum (Al)	0.003	0.47	0.0543	0.391	50	1400	740	508	0.6	55.4	82.6
Antimony (Sb)	0.0001	0.00019	0.00024	0.00023	0.1	<0.10	<0.10	<0.10	0.01	<0.010	0.016
Arsenic (As)	0.0001	0.00337	0.00396	0.00394	0.1	0.91	0.84	0.35	0.01	0.18	0.234
Barium (Ba)	0.0001	0.0376	0.0347	0.0447	0.5	15.8	6.78	3.61	0.02	38.9	60.4
Beryllium (Be)	0.0001	<0.00010	<0.00010	<0.00010	0.1	<0.10	<0.10	<0.10	0.01	<0.010	<0.010
Bismuth (Bi)	0.00005	<0.000050	<0.000050	<0.000050	0.2	<0.20	<0.20	<0.20	0.004	<0.0040	<0.0040
Boron (B)	0.01	0.03	0.035	0.052	5	6.9	<5.0	<5.0	0.4	0.99	1
Cadmium (Cd)	0.000005	0.0000168	0.0000141	0.0000215	0.02	0.069	0.035	<0.020	0.004	0.123	0.126
Calcium (Ca)	0.05	39.6	40.1	43.8	50	87300	18400	5640	40	135000	214000
Cesium (Cs)	0.00001	0.00007	0.00001	0.000077	ND	ND	ND	ND	0.004	0.0093	0.0137
Chromium (Cr)	0.0001	0.0009	0.00018	0.00069	0.5	3.41	1.77	1.1	0.04	0.166	0.59
Cobalt (Co)	0.0001	0.00042	0.00011	0.00036	0.1	2.05	1.24	0.4	0.02	0.117	0.14
Copper (Cu)	0.0005	0.00283	0.00229	0.00261	0.5	1.77	0.59	0.57	0.04	1.64	2.02
Iron (Fe)	0.01	0.585	0.06	0.548	50	2930	1410	944	1	91.5	129
Lead (Pb)	0.00005	0.00042	0.000078	0.000361	0.5	1.48	0.86	0.58	0.008	0.0935	0.116
Lithium (Li)	0.001	0.0193	0.0227	0.027	2	4.2	<2.0	<2.0	0.1	0.26	0.31
Magnesium (Mg)	0.005	25.5	28.1	29.9	20	42600	5380	759	2	133	176
Manganese (Mn)	0.0001	0.0329	0.0329	0.0493	1	218	58.2	16.3	0.02	33.7	55.1
Molybdenum (Mo)	0.00005	0.0012	0.00148	0.00175	0.1	<0.10	<0.10	<0.10	0.02	0.05	0.068
Nickel (Ni)	0.0005	0.00254	0.00223	0.00294	0.5	3.58	1.74	0.98	0.04	0.884	1.42
Potassium (K)	0.05	4.87	5.06	6.39	100	330	100	76	4	84.1	144
Phosphorus (P)	0.03	0.171	0.161	0.197	50	177	257	<100	4	522	739
Rubidium (Rb)	0.0002	0.00292	0.00196	0.00295	ND	ND	ND	ND	ND	ND	ND
Selenium (Se)	0.00005	0.000353	0.000402	0.000408	0.2	<0.20	<0.20	<0.20	0.04	0.217	0.236
Silicon (Si)	0.1	5.89	3.41	6.36	ND	ND	ND	ND	ND	ND	ND
Silver (Ag)	0.00001	<0.000010	<0.000010	0.00001	0.1	<0.10	<0.10	<0.10	0.02	<0.020	<0.020
Sodium (Na)	0.05	23	26.6	36.6	50	73	<50	<50	4	999	1550
Strontium (Sr)	0.0002	0.141	0.184	0.178	0.5	35.5	13.6	5.39	0.1	196	305
Sulfur (S)	0.5	27.2	33.7	37.3	1000	<1000	<1000	<1000	20	431	642
Tellurium (Te)	0.0002	<0.00020	<0.00020	<0.00020	0.1	<0.10	<0.10	<0.10	0.04	<0.040	<0.040
Thallium (Tl)	0.00001	0.000014	0.000011	0.000015	0.05	<0.050	0.057	<0.050	0.006	<0.0060	<0.0060
Thorium (Th)	0.0001	0.00012	<0.00010	<0.00010	ND	ND	ND	ND	0.01	0.021	0.039
Tin (Sn)	0.0001	<0.00010	<0.00010	<0.00010	2	<2.0	<2.0	<2.0	0.02	<0.020	<0.020
Titanium (Ti)	0.0003	0.019	0.00152	0.0108	1	89.6	102	33	0.03	1.89	2.81
Tungsten (W)	0.0001	<0.00010	<0.00010	<0.00010	0.5	<0.50	<0.50	<0.50	ND	ND	ND
Uranium (U)	0.00001	0.00198	0.00234	0.0021	0.05	0.381	0.201	0.089	0.002	0.0648	0.0666
Vanadium (V)	0.0005	0.0038	0.00274	0.00405	0.2	4.43	2.49	1.33	0.01	0.572	0.635
Zinc (Zn)	0.003	<0.0030	<0.0030	<0.0030	2	8.1	5.6	<2.0	0.2	5.13	6.11
Zirconium (Zr)	0.0002	0.00055	<0.00020	0.00046	1	1.2	<1.0	<1.0	0.6	<0.60	<0.60

Levels of each element determined via ICP. ND = not determined; Samples measured in µg/ mL, µg/g, & µg/g wet weight, for water, sediment, and whole zebra mussel tissues, respectively. Detection limit listed for each sample type: water, sediment, and tissue.

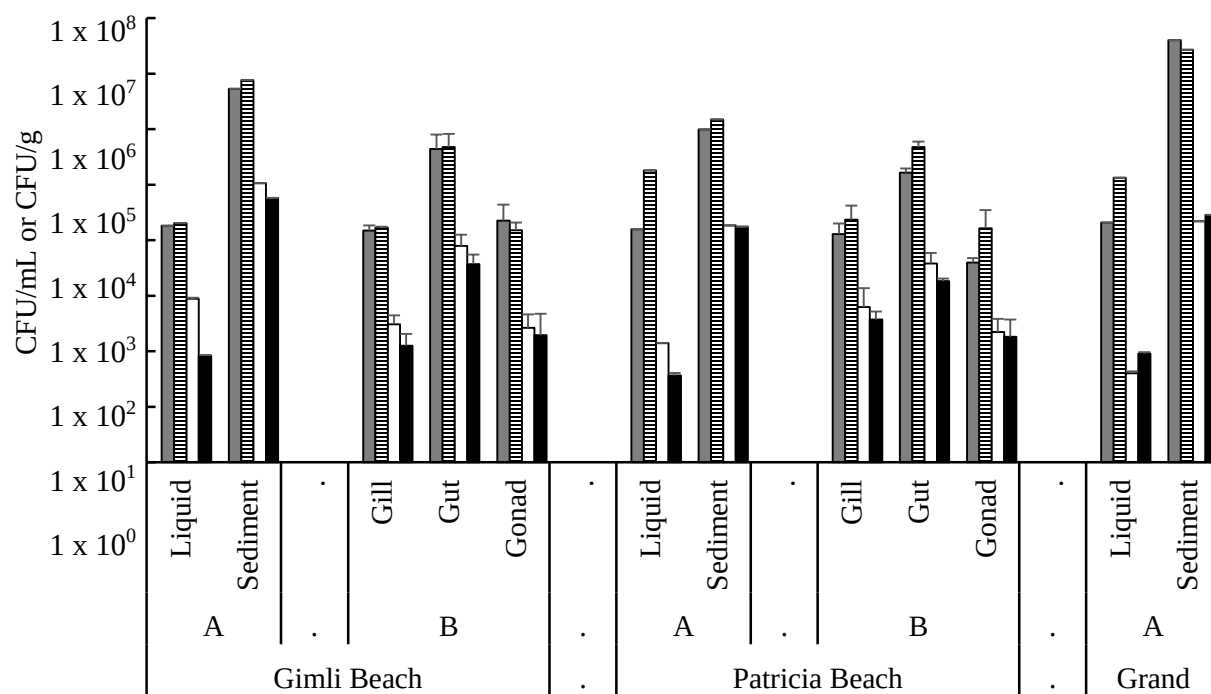


Fig. 3.2. Total culturable microorganisms observed at both Gimli Beach and Patricia Beach. Enumerations of microorganisms cultivated on different media were grouped by sample type into (A) Environmental sediment and water samples, and (B) Zebra mussel gill, gut and gonad tissues taken from three averaged animals. Bacterial counts were recovered from samples among four types of media, in triplicate; RO, grey; BG-11; striped; PNSM, white; and RO_T, black.

Table 3.2. The AAP population cultured from the environment and zebra mussel tissues.

Sample	Medium	# Pigmented isolates tested	#AAP	% AAP of Pigmented isolates	% AAP of Total	AAP CFU/mL or CFU/g
Water	RO	94	4	4.26	1.74	3.17×10^2
	RO _T	27	4	14.81	15.39	1.08×10^1
Sediment	RO	125	8	6.40	13.98	2.17×10^6
	RO _T	25	6	24.00	18.72	6.49×10^3
Gill	RO	153	4	2.61	0.48	6.64×10^1
	RO _T	17	0	0.00	0.00	ND
Gut	RO	195	7	3.59	1.15	3.48×10^3
	RO _T	27	1	3.70	3.86	1.06×10^2
Gonad	RO	96	1	1.04	0.16	2.12×10^1
	RO _T	2	0	0.00	0.00	ND

Table 3.3. Enumeration of culturable microorganisms from each sampling site and zebra mussel organ on selective media.

Site	Sample	RO		PNS _M		RO _T		BG-11		
		Total	Pigmented	Total	Pigmented	Total	Pigmented	Total	Pigmented	AOP
B1	Water	1.81 x10 ⁴	4.23 x10 ¹	8.83 x10 ²	ND	8.33 x10 ¹	8.33 x10 ¹	2.02 x10 ⁴	1.13 x10 ²	6.67 x10 ⁰
B1	Sediment	5.35 x10 ⁶	1.83 x10 ⁴	1.07 x10 ⁵	5.00 x10 ⁰	5.80 x10 ⁴	5.48 x10 ²	7.60 x10 ⁶	1.07 x10 ⁴	5.67 x10 ³
B2	Water	1.58 x10 ⁴	1.25 x10 ²	1.40 x10 ²	ND	3.67 x10 ¹	3.67 x10 ¹	1.80 x10 ⁵	1.52 x10 ³	1.33 x10 ⁴
B2	Sediment	9.91 x10 ⁵	7.44 x10 ³	1.84 x10 ⁴	ND	1.76 x10 ⁴	1.62 x10 ²	1.49 x10 ⁶	4.24 x10 ³	1.00 x10 ⁴
B3	Water	2.08 x10 ⁴	8.73 x10 ¹	4.00 x10 ¹	ND	9.00 x10 ¹	9.00 x10 ¹	1.33 x10 ⁵	1.09 x10 ³	2.73 x10 ⁴
B3	Sediment	4.02 x10 ⁷	1.52 x10 ⁵	6.29 x10 ³	ND	8.14 x10 ³	2.50 x10 ²	7.71 x10 ⁶	5.17 x10 ⁴	3.81 x10 ²
B1-Z1	Gill	1.07 x10 ⁴	3.50 x10 ¹	1.83 x10 ²	ND	9.16 x10 ¹	9.16 x10 ¹	1.58 x10 ⁴	3.53 x10 ¹	ND
B1-Z1	Gut	9.41 x10 ⁵	3.47 x10 ³	5.78 x10 ³	ND	4.00 x10 ³	3.26 x10 ¹	9.62 x10 ⁵	2.79 x10 ³	ND
B1-Z1	Gonad	4.20 x10 ³	1.67 x10 ¹	ND	ND	ND	ND	7.13 x10 ³	1.32 x10 ¹	ND
B1-Z2	Gill	1.89 x10 ⁴	7.03 x10 ¹	2.41 x10 ²	ND	4.82 x10 ¹	4.82 x10 ¹	1.75 x10 ⁴	3.37 x10 ¹	ND
B1-Z2	Gut	1.24 x10 ⁵	4.64 x10 ²	3.65 x10 ³	ND	1.27 x10 ³	5.53 x10 ⁰	2.20 x10 ⁵	5.31 x10 ²	ND
B1-Z2	Gonad	5.22 x10 ⁴	1.54 x10 ²	4.79 x10 ²	ND	5.86 x10 ²	1.60 x10 ⁰	2.12 x10 ⁴	3.94 x10 ¹	ND
B1-Z3	Gill	1.54 x10 ⁴	7.56 x10 ¹	4.93 x10 ²	ND	2.35 x10 ²	2.35 x10 ⁰	1.71 x10 ⁴	4.18 x10 ¹	ND
B1-Z3	Gut	2.53 x10 ⁵	9.33 x10 ²	1.43 x10 ⁴	ND	5.77 x10 ³	4.48 x10 ¹	2.42 x10 ⁵	3.40 x10 ²	ND
B1-Z3	Gonad	1.12 x10 ⁴	4.30 x10 ¹	3.11 x10 ²	ND	ND	ND	1.67 x10 ⁴	2.59 x10 ¹	ND
B2-Z1	Gill	2.21 x10 ⁴	1.15 x10 ¹	2.04 x10 ²	ND	3.56 x10 ²	3.56 x10 ⁰	1.55 x10 ⁴	3.00 x10 ¹	ND
B2-Z1	Gut	1.99 x10 ⁵	3.85 x10 ²	5.84 x10 ³	1.17 x10 ¹	2.10 x10 ³	2.10 x10 ¹	4.23 x10 ⁵	9.81 x10 ²	ND
B2-Z1	Gonad	3.70 x10 ³	1.68 x10 ⁰	5.61 x10 ¹	ND	5.61 x10 ¹	5.61 x10 ¹	2.81 x10 ³	5.05 x10 ⁰	ND
B2-Z2	Gill	1.13 x10 ⁴	1.63 x10 ¹	1.68 x10 ³	ND	5.60 x10 ²	5.60 x10 ⁰	4.89 x10 ⁴	1.17 x10 ²	ND
B2-Z2	Gut	1.25 x10 ⁵	2.10 x10 ²	4.67 x10 ³	ND	1.81 x10 ³	1.81 x10 ¹	3.64 x10 ⁵	6.65 x10 ²	ND
B2-Z2	Gonad	3.20 x10 ³	6.73 x10 ⁰	1.68 x10 ²	ND	4.49 x10 ²	3.37 x10 ⁰	3.54 x10 ³	8.42 x10 ⁰	ND
B2-Z3	Gill	5.10 x10 ³	1.35 x10 ¹	ND	ND	1.99 x10 ²	1.19 x10 ⁰	5.46 x10 ³	1.27 x10 ¹	ND
B2-Z3	Gut	1.73 x10 ⁵	5.85 x10 ²	9.14 x10 ²	ND	1.55 x10 ³	1.55 x10 ¹	6.45 x10 ⁵	2.19 x10 ³	ND
B2-Z3	Gonad	5.01 x10 ³	7.48 x10 ⁰	4.40 x10 ²	ND	4.40 x10 ¹	4.40 x10 ¹	4.27 x10 ⁴	7.04 x10 ¹	ND

ND = None Detected. B1, B2, & B3 water sample counts are in CFU/mL; all remaining are in CFU/g. All CFU values shown are the mean of triplicates. The last column depicts the CFU of detected Chl a containing AOP from the BG-11 medium.

gonad tissues from both sites. Furthermore, AAP were detected on RO_T primarily from water and sediment, and in a higher proportion compared to other cultivated heterotrophs in the same medium. No AAP or PNS were successfully isolated from any site or tissue samples on PNSM or BG-11. Of note, AOP were only cultivated from environmental samples, with none coming from animal tissues.

3.5.3. Bacterial community identified by culture-independent ribosomal 16S rDNA sequencing

The rDNA sequenced from each site via Illumina MiSeq has been deposited on the National Center for Biotechnology Information Sequence Read Archive as BioProject PRJNA603112. Through the Qiime2 pipeline, forward and reverse reads were combined to produce up to 300,000 trimmed 16S V4 regions over 291 bp long for binning. Using the Silva 132 database for taxonomic relations matching by 99%, no less than 501 separate genetic relatives to type species were found. Once similar reads were binned and identified from the reference database, relative quantities of phylons could be estimated for each sample and site (Fig. 3.3). Diversity of species was statistically quantified via Faith's phylogenetic diversity (PD) (Faith *et al.*, 1987), to observe that bacterial communities from zebra mussel tissues were less genetically diverse than in the surrounding environment (Fig. 3.4). Furthermore, water and sediment both had similar bacterial species richness, which was significantly higher than in all tissues (Table 3.4). Among the bivalve microflora, the gut-associated microbial community had the largest range in bacterial species diversity, statistically different from that of the gonadal tissues. In addition, the microbial composition formed among gonads had the least variation in species, as well as the smallest variety overall. Jaccard-Emperor based β -diversity, a qualitative measure of community dissimilarity, divulged separation of samples predominantly by origin: environmental vs. animal (Fig. 3.7, shape), but also by site (Fig. 3.7, colour), and confirmed tissue communities to be similar within biological replicates. The differentiation of gut and gonad bacterial representatives defined in α -diversity was also present in the β -diversity plot. The gill associated bacterial community shared similarities to both gut and gonad tissue cohorts, which was statistically supported in Faith's PD plot (Fig. 3.4).

Since the greatest divergence in sequenced bacterial communities existed between environmental samples and the inhabitants found among all zebra mussel tissues (Fig. 3.3 and 3.4), we grouped all 6 environmental vs. all 18 animal samples (Fig. 3.5) to discuss the key bacterial community members found in either case. The dissimilar proportions existed at the phylum (Fig. 3.3) and lower levels (Fig. 3.6). Phyla *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*,

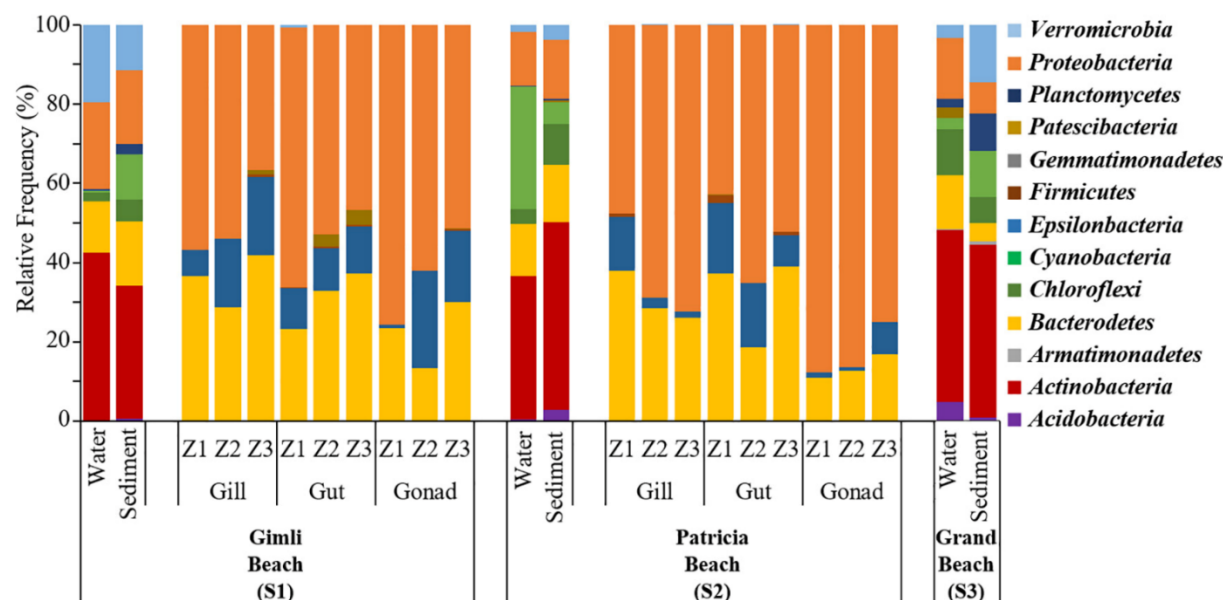


Fig. 3.3. Stacked bar charts representing bacterial community structure of each sample. Phyla composition for each sample were determined by eDNA amplicon 16S V4 rDNA sequencing via Illumina MiSeq. All sites featured up to 501 bacterial species from a library size of over 174,904 genomic reads per sample. Bacterial communities from water and sediment were sequenced. In addition, three adult zebra mussels were dissected (Z1, Z2, Z3), and bacterial communities from separate organs (gill, gut, and gonad) were sequenced.

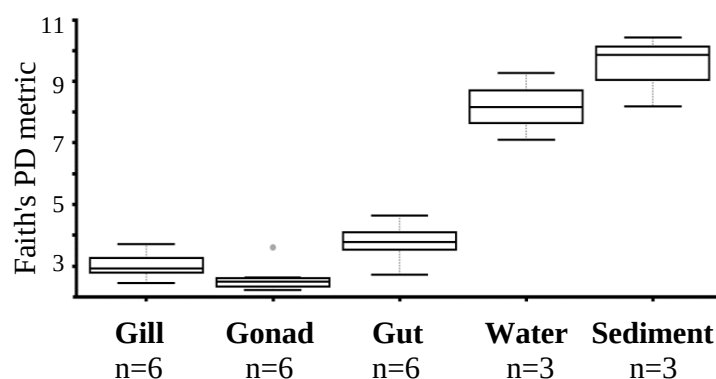


Fig. 3.4. Faith's Phylogenetic Diversity (PD) plot of species α -diversity richness. Higher distance represented a larger diversity of species found in samples, as well as differentiation of bacterial community composition. Gill, gut, and gonad tissues each included 6 individual biological replicates (n), 3 from both Gimli and Patricia Beaches. Both water and sediment samples contained 3 biological replicates, one from each Gimli Beach, Patricia Beach, and Grand Beach.

Table 3.4. Community diversity richness among bivalve microbiome. Determined with α -rarefaction diversity statistics shown in Fig. 4 Faith's PD.

Group 1	Group 2	H	p-value	q-value
gill (n=6)	gonad (n=6)	3.102564103	0.078169086	0.08685454
gill (n=6)	gut (n=6)	3.102564103	0.078169086	0.08685454
gill (n=6)	liquid (n=3)	5.4	0.020136752	0.028766788
gill (n=6)	sediment (n=3)	5.4	0.020136752	0.028766788
gonad (n=6)	gut (n=6)	6.564102564	0.010405620	0.028766788
gonad (n=6)	liquid (n=3)	5.4	0.020136752	0.028766788
gonad (n=6)	sediment (n=3)	5.4	0.020136752	0.028766788
gut (n=6)	liquid (n=3)	5.4	0.020136752	0.028766788
gut (n=6)	sediment (n=3)	5.4	0.020136752	0.028766788
liquid (n=3)	sediment (n=3)	2.333333333	0.126630458	0.126630458

H = difference in species richness, n = population size, p-value = probability, q-value = adjusted p-value. P-values of less than .05, in bold, confirm separation of community diversity, where above .05 represents samples that are more similar to one another.

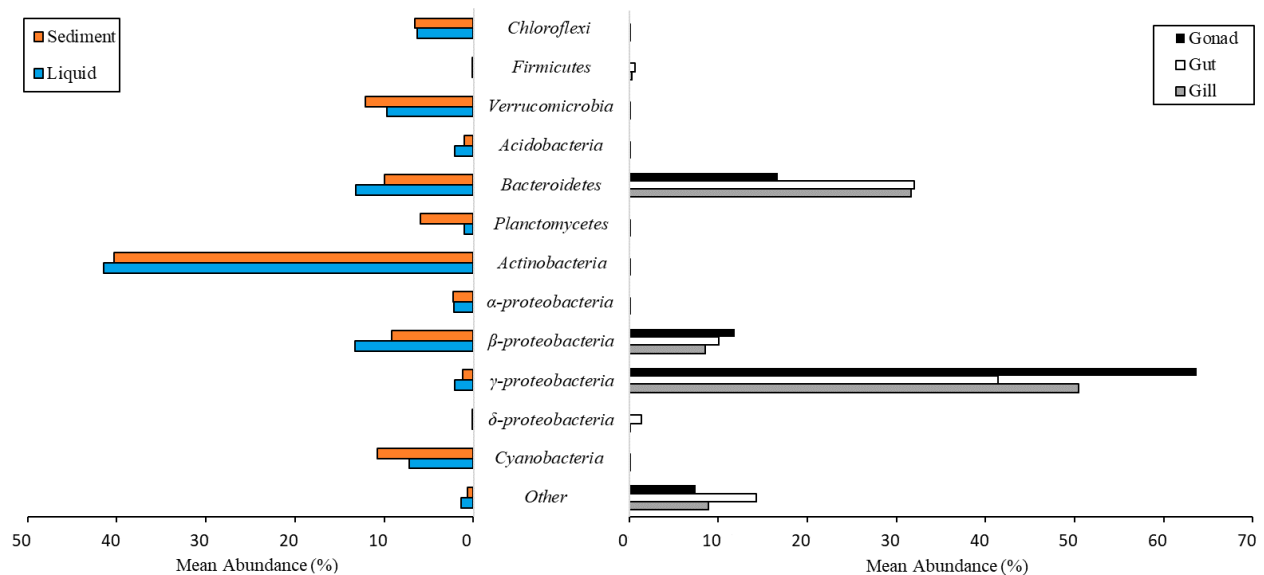


Fig. 3.5. Phyla Composition of Microbial Communities associated with zebra mussel gill, gut and gonad, and compared to environmental water and sediment. Sequences are grouped by phylotype, shown in the center. All data represent 16S V4 rDNA sequences, mean of samples from 3 beaches for water and sediment, and mean of 6 samples for each gill, gut, and gonad, from 2 beaches both having 3 mussels examined. “Other” contains phyla *Armatimonadetes*, *Epsilonbacteraeota*, *Gemmatimonadete* and *Patescibacteria*.

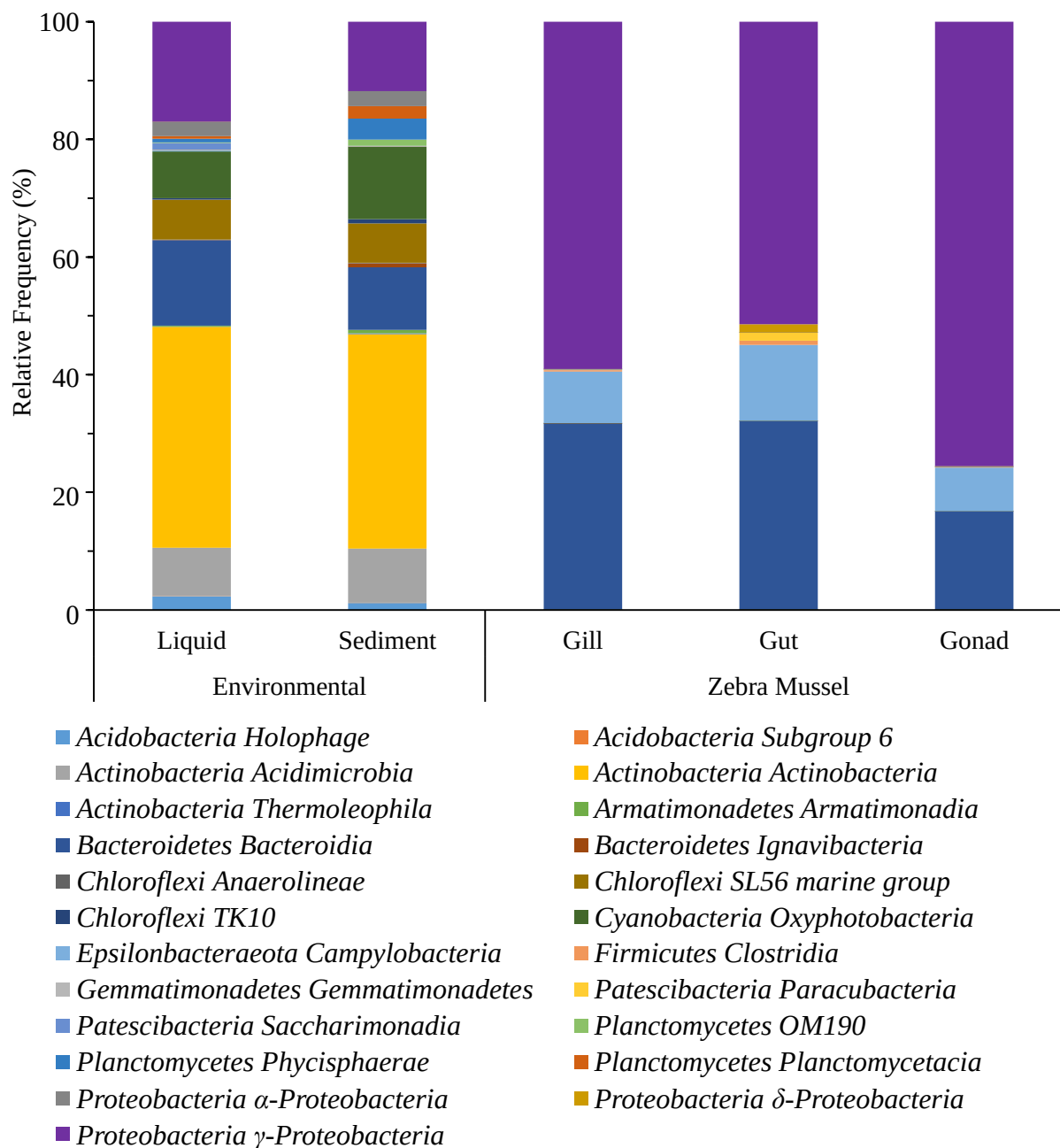


Fig. 3.6. Sequenced eDNA of 16S V4 rDNA grouped by sample type and depicted at the as classes of phyla.

Planctomycetes, and *Verrucomicrobia* were present practically exclusively within environmental samples. When combined, they made up to ~73.2% of the population, while only ~0.7% in all tissues. Inversely, *Epsilonbacteraeota* and *Firmicutes* were localized primarily in the mussel tissues as ~5.6% of the community, and less than ~0.1% in environment. Common for bivalves and their habitat were phyla *Bacteroidetes*, *Patescibacteria*, and *Proteobacteria*, which made up to ~11.6, ~0.6, and ~15.1%, respectively at environmental sites, and higher in bivalve tissues, reaching ~27.5, ~0.5, and ~60.7%. Division of the phyla into genetic classes (Fig. 3.6), revealed that most *Bacteroidetes* were *Bacteroidia*, and *Bacteriales* were associated with zebra mussel tissues only, whereas the classes *Chitinophagales*, *Shingobacteriales*, *Cytophagales*, and *Ignavibacteria* were exclusively environmental representatives at less than 0.3% of the population. *Patescibacteria* were more divergent, as the local environment only contained *Saccharimonadia*, while zebra mussels only had *Parcubacteria*. Interestingly, α -*Proteobacteria* comprised 2.2% of the environmental population, and only 0.02% in mussel tissues. Less than 0.02% represented δ -*Proteobacteria* in the environment, and 0.5% were within the animals. Unexpectedly, γ -*Proteobacteria* constituted 12.7% of environmental community and a striking 61.0% in bivalves. *Flavobacteriales* were found in all samples studied. Within γ -*Proteobacteria*, *Pseudomonas* were predominant in the mussels, while only slightly >2% in the environment. *Aeromonadales* and *Alteromonadales* were among the zebra mussel community, while *Xanthomonadales* were strictly within the local habitat. β -*Proteobacteria* were encountered similarly in all specimens.

To examine the AAP population in the 16S V4 rDNA sequenced community, we have considered all published AAP to date, Table 1.2. These included genera from the family *Sphingomonadaceae* within the *Sphingomonadales* order of α -*Proteobacteria*. In addition, AAP genera were described within *Rhodobacteraceae* of the order *Rhodobacterales* of α -*Proteobacteria*, and genera in the family *Acetobacteraceae* of the *Rhodospirillales* order of α -*Proteobacteria*. We also included genera of the α -*Proteobacteria* *Hoeflea* and *Roseateles*, β -*Proteobacteria* *Rhodoferrax*, *Limnohabitans* and *Aquicola*, and *Congregibacter* and *Chromocurvus* of γ -*Proteobacteria*. With these considerations, littoral water samples contained a 16S V4 rDNA sequenced community with 2.78% representing AAP, while around 0.88% of the microbial genes belonged to AAP among littoral sediment. When pooled by organ, mussel samples had 0.22, 1.13 and 0.20% of the total 16S genes annotated as AAP from the gill, gut, and gonad tissues, respectively. In comparison, 13.26 and 17.08% of the respective lake water and sediment sequenced community hailed from cyanobacteria, while zebra mussel tissues revealed no oxygenic phototrophic DNA present.

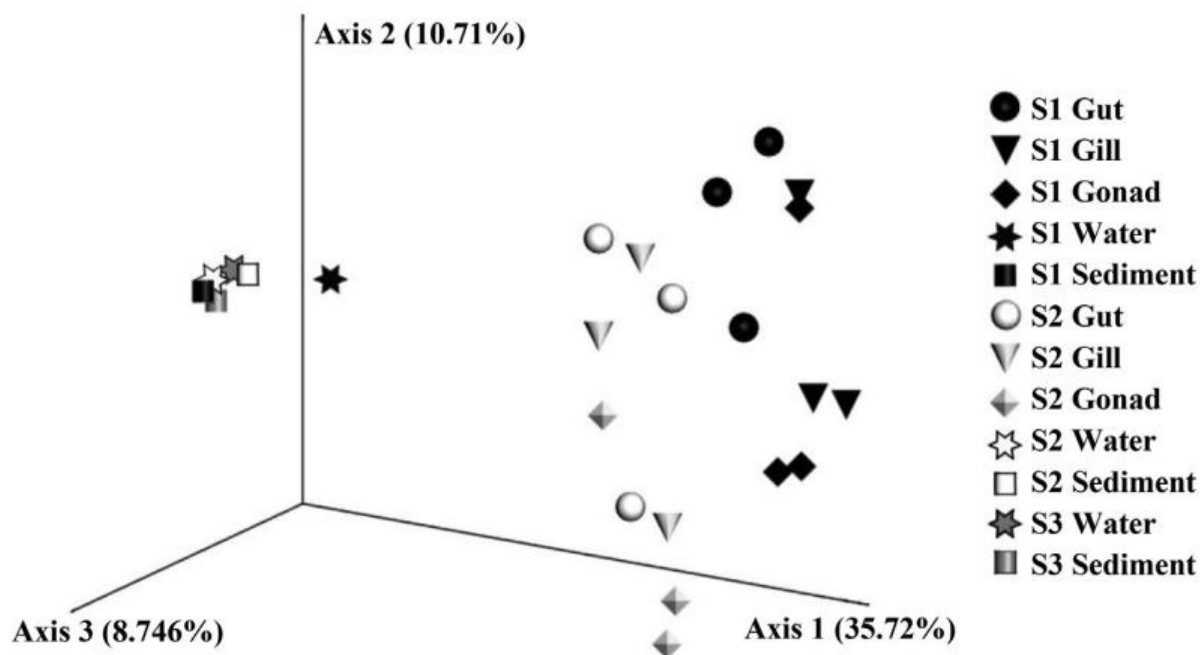


Fig. 3.7. Jaccard Emperor β -diversity of samples. A qualitative measure of community dissimilarity depicted diversity of species found in sequenced 16S V4 rDNA of eDNA from all samples, by applying each individual bacterial community to an ordinance with only metrics (x, y, z) rather than numerous values. Individual bacterial communities have unique shades to differentiate site of origin: S1, black; S2, white; S3, grey. The same data is separated in accordance to sample type by shape. Gut samples are spheres, gill are cones, gonad are diamonds, water are stars, and sediment are squares. In this way, environmental communities can be identified from all other zebra mussel tissue samples.

3.5.4. AAP isolated from zebra mussel tissues

Each purified pigmented strain from bivalve tissue that was confirmed to produce BChl *a* aerobically, had 16S rDNA sequenced and compared to known type species for identification (Table 3.5). Sequences for each AAP isolate, Z1, Z2, Z5, Z6, Z7, Z24, Z27, Z39, Z59, and Z68 were deposited on GenBank as MN987006 – MN987015, respectively. Relation of strains to a range of genera containing AAP species was depicted in a phylogenetic tree (Fig. 3.8). All AAP isolates had genomic 16S rDNA sequence similarity to the family members of *Sphingomonadaceae*, specifically *Porphyrobacter* and *Erythromicrobium*, in the range between 98.7 – 99.6%. Based on these values, they may represent new species or strains, therefore further clarification of lineage was performed with the maximum likelihood method and 10,000 bootstrap iterations, specifically among several known *Porphyrobacter* and *Erythromicrobium* (Fig. 3.9). All of our isolates were orange or red-orange in colour, producing carotenoids absorbing in the range 410-595 nm, along with typical reaction center and light harvesting complexes with major absorption peaks between 800-875 nm (Yurkov and Beatty, 1998). Cellular morphology was examined with 1 and 3 day old cultures, revealing motility and short rods <2 µm in most early phases of growth, and elongated cells >4 µm or filaments >10 µm within aged cultures (Fig. 3.10). Taxonomical tests were performed (Table 3.6) and revealed notable AAP features (Yurkov and Beatty, 1998), including lack of anaerobic growth for all strains, and utilization of numerous carbon sources. Many of them demonstrated psychrotrophy and grew optimally at around 15 °C, according to defined temperature range preferences for growth (Wiegel, 1990). Most were susceptible to a majority of antibiotics tested.

Representatives from each AAP taxon (Fig. 3.9), were grown in the presence of increasing levels of K₂TeO₃ and compared to oxide-free grown cultures to determine MIC (Fig. 3.11). In this way, strain Z1 was inhibited by 50 µg/mL, Z7 and Z39 by 100 µg/mL, whereas Z2, Z24, Z59, and Z68, by 200 µg/mL. All but Z1 had an increase in growth at sub-inhibitory concentrations, 2-fold or greater OD at 25 µg/mL for Z2, Z7, Z24, Z39, Z59, and Z68, relative to cultures without tellurite addition.

3.6. Discussion

3.6.1. Study site description

Sites were chosen on the littoral shores of Lake Winnipeg's South Basin, as they have had significant algal blooms in the past, likely containing a diverse microbial community (MWSD, 2011; MARD, 2020). The lake's current eutrophic and oxic nature made it an ideal location to study AAP, specifically in the surface euphotic zone, as the water appeared quite turbid upon sampling, suggesting high organic content and

Table 3.5. Polyphasic analysis of representative AAP isolated from zebra mussel tissues.

Site	Strain	In vivo crt* peaks (nm)	In vivo BChl <i>a</i> peaks (nm)	Colour	Cell shape	16S rRNA sequence (bp)	16S rRNA partial gene sequence similarity (%) to type species
S1-Z1 Gut	Z1	464, 490	870	Orange	Ovoid rods, chains	1396	99.1, <i>Porphyrobacter colymbi</i>
S1-Z2 Gill	Z2	485, 797	820, 867	Red-orange	Ovoid rods, chains	1404	99.4, <i>Porphyrobacter tepidarius</i>
S2-Z3 Gill	Z5	462, 797	822, 866	Red-orange	Rods, chains	1399	99.6, <i>Porphyrobacter tepidarius</i>
S1-Z1 Gut	Z6	419, 452, 493 580	801, 871	Orange	Long rods	1395	99.3, <i>Porphyrobacter neustonensis</i>
S2-Z3 Gonad	Z7	462, 481	871	Orange	Short curved rods	1393	99.6, <i>Erythromicrobium ramosum</i>
S2-Z3 Gut	Z24	460, 483	869	Orange	Rods	1404	98.7, <i>Porphyrobacter sanguineus</i>
S1-Z1 Gut	Z27	452, 483, 590	800, 871	Orange	Ovoid rods, chains	1396	99.4, <i>Porphyrobacter neustonensis</i>
S2-Z3 Gut	Z39	455, 478	867	Orange	Ovoid rods	1407	98.9, <i>Porphyrobacter sanguineus</i>
S1-Z3 Gut	Z59	460, 491	870	Orange	Ovoid rods	1401	99.5, <i>Porphyrobacter neustonensis</i>
S1-Z1 Gut	Z68	455, 481, 592	801, 871	Orange	Ovoid rods	1396	99.4, <i>Porphyrobacter neustonensis</i>

S1 and S2 were Gimli and Patricia Beaches, respectively; Z1, Z2 and Z3 were zebra mussel replicates, each with gill, gut, or gonad tissue isolates; *crt = carotenoids.

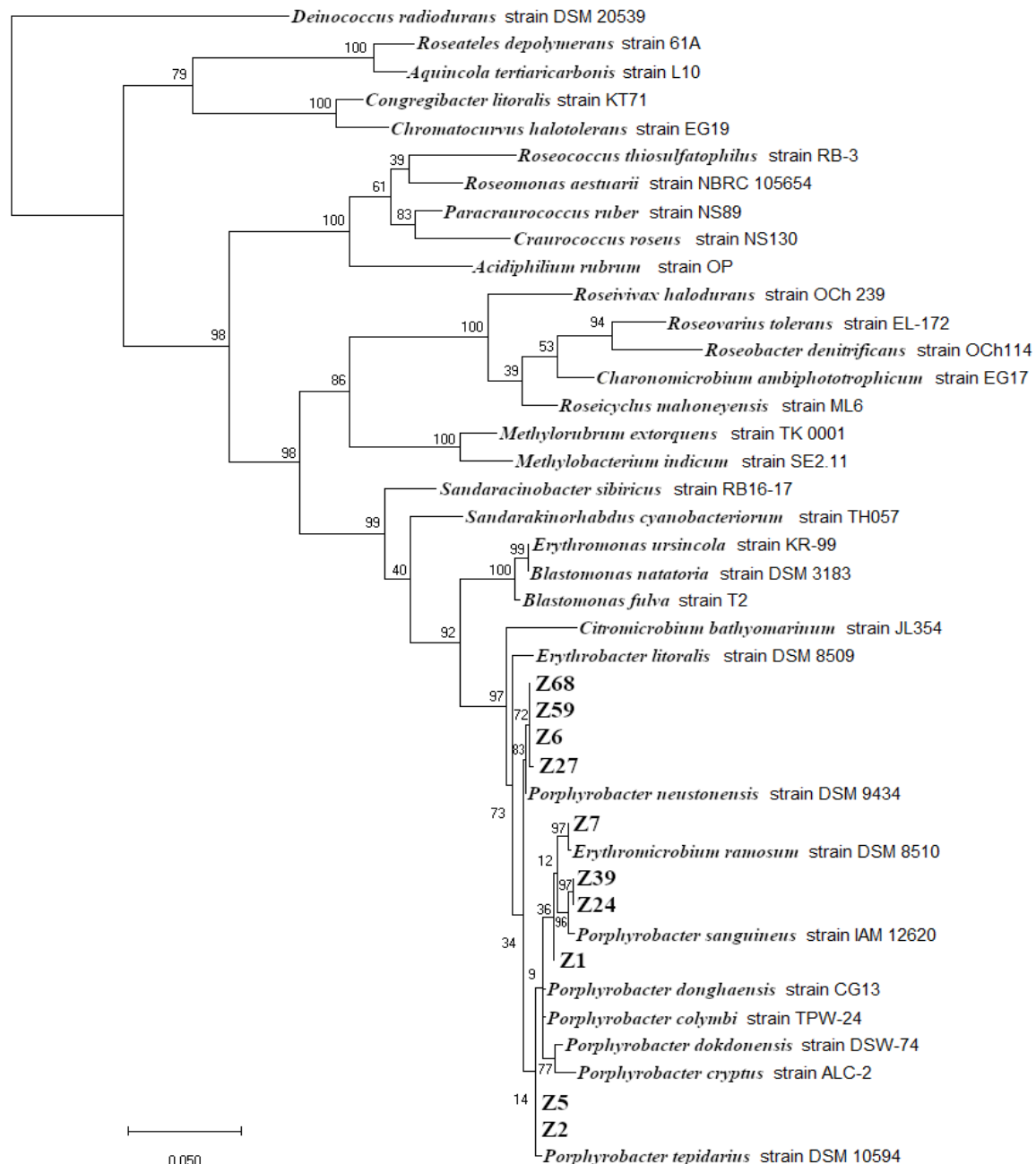


Fig. 3.8. A 16S rRNA gene sequence phylogenetic analysis of zebra mussel isolated AAP. 16S rRNA partial gene sequences compared to closest relatives and additional known AAP genera by Maximum Likelihood method. The tree with the highest log likelihood (-10774.56) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, as shown with scale bar. The analysis involved 42 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1269 positions in the final dataset.

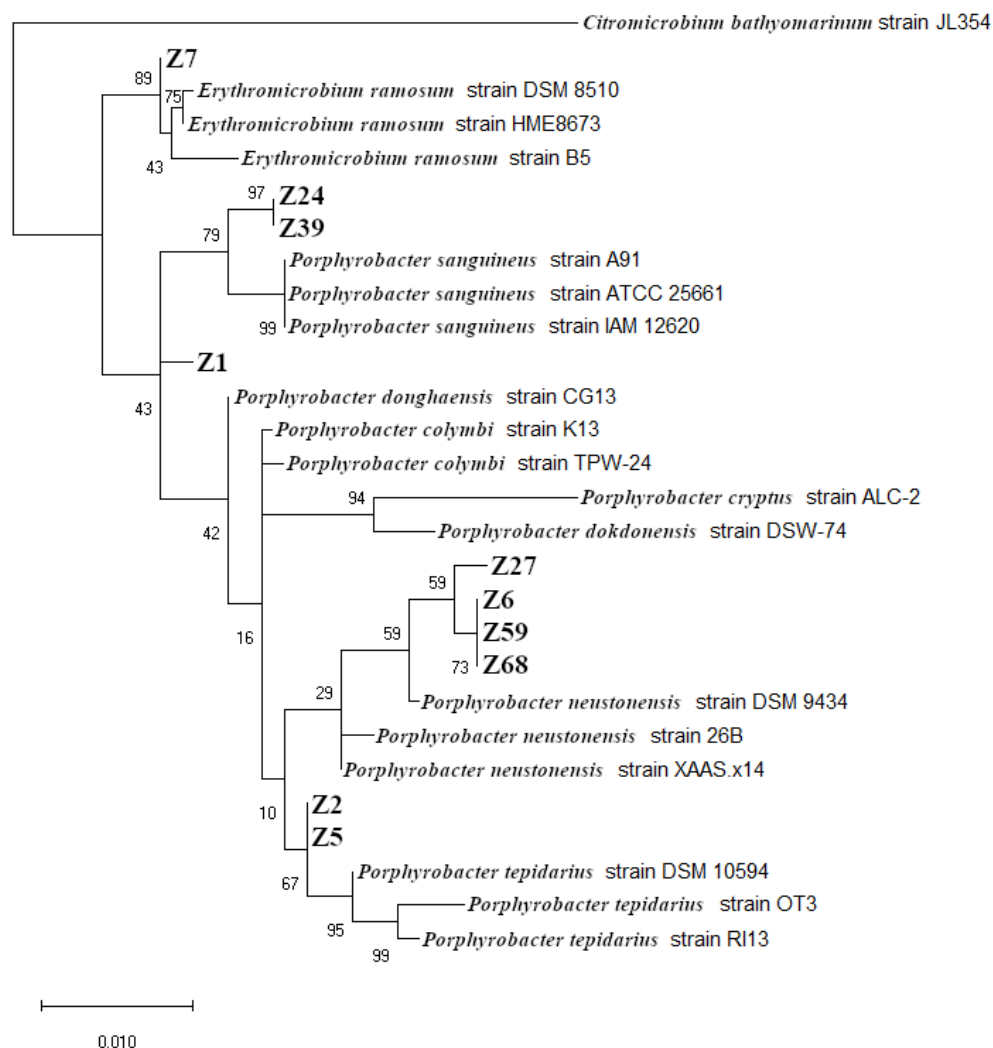


Fig S8.9. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (-3088.14) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1326 positions in the final dataset.

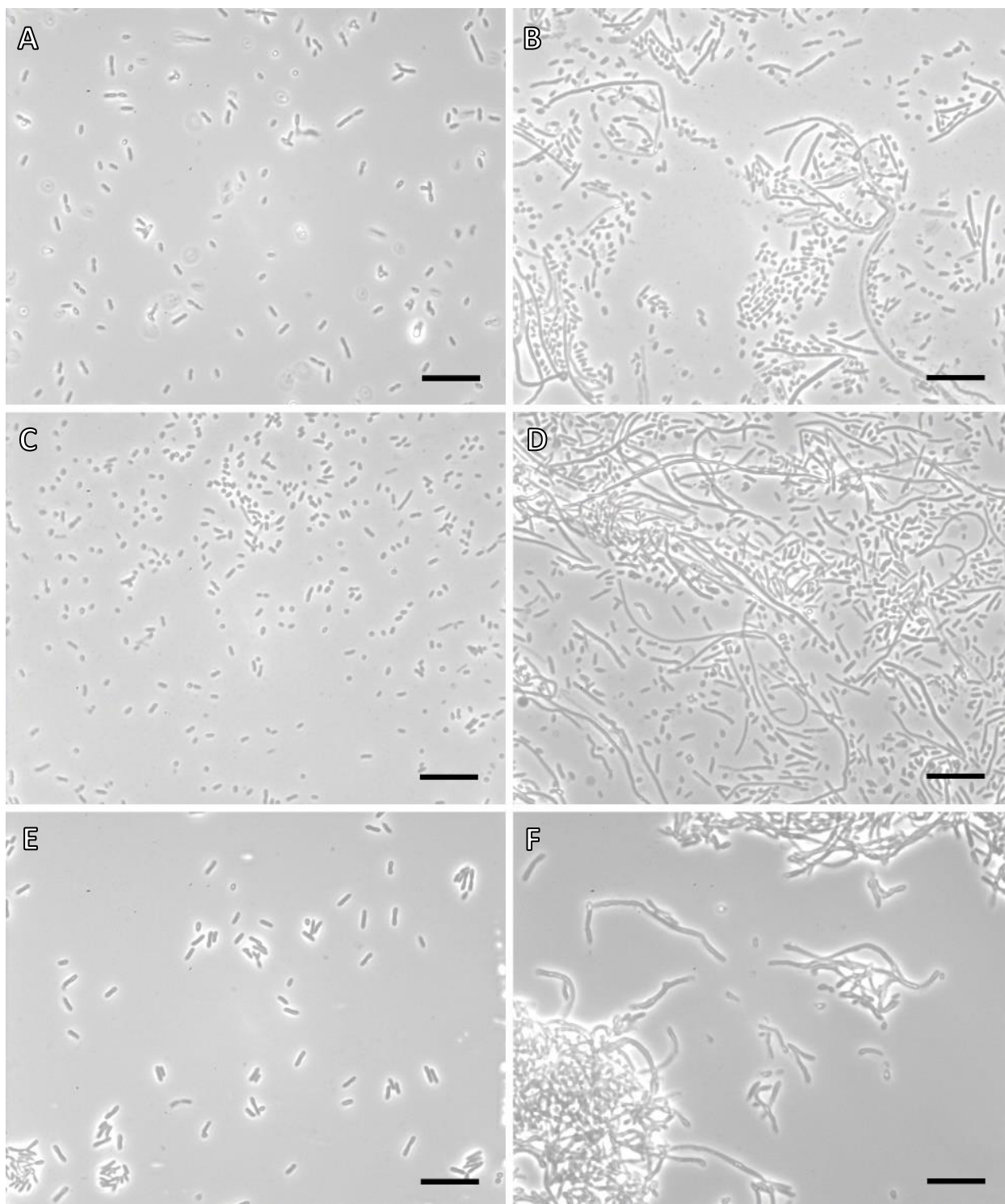


Fig. 3.10. Micrographs of zebra mussel AAP isolates during early and late growth phases. (A, C, E) Morphology of one-day old incubated strains Z1, Z2, and Z6, respectively. **(B, D, F)** After three days of growth, all depicted extended cell length morphology. Scale bar is 10μm for all micrographs.

Table 3.6. Expansive physiological tests performed with AAP isolates from zebra mussel tissues.

Strain	Z1	Z2	Z5	Z6	Z7	Z27	Z24	Z39	Z59	Z68
Gram stain	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	+	-	-	+	+	+	+
16S rDNA sequence similarity:										
Read Length	1396 99.1%	1404 99.4%	1399 99.6%	1395 99.3%	1393 99.6%	1396 99.4%	1404 98.7%	1407 98.9%	1401 99.5%	1396 99.4%
Most related type species	<i>Porphyrobacter colymbi</i> 99.0%	<i>Porphyrobacter tepidarius</i> 99.2%	<i>Porphyrobacter tepidarius</i> 99.4%	<i>Porphyrobacter neustonensis</i> 98.7%	<i>Erythromicrobiu m ramosum</i> 98.6%	<i>Porphyrobacter neustonensis</i> 98.7%	<i>Porphyrobacter sanguineus</i> 98.5%	<i>Porphyrobacter sanguineus</i> 98.6%	<i>Porphyrobacter neustonensis</i> 98.8%	<i>Porphyrobacter neustonensis</i> 98.9%
Second most related species	<i>Porphyrobacter donghaensis</i>	<i>Porphyrobacter colymbi</i>	<i>Porphyrobacter colymbi</i>	<i>Erythromicrobi um ramosum</i>	<i>Porphyrobacter donghaensis</i>	<i>Porphyrobacter donghaensis</i>	<i>Porphyrobacter donghaensis</i>	<i>Porphyrobacter donghaensis</i>	<i>Erythromicrobiu m ramosum</i>	<i>Erythromicrobiu m ramosum</i>
Enzyme Activities:										
Catalase	-	+	+	+	+	+	+	-	+	+
Oxidase	+	+	-	+	+	+	+	+	+	+
Nitrate Reductase	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Hydrolysis of:										
Agar	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-
Tween 60 (Lipase activity)	+	+	+	+	+	+	+	+	+	+
Utilization of:										
Acetate	+	+	+	-	+	+	+	+	-	+
Bactopeptone	+	+	+	+	+	+	+	+	+	+
Butyrate	+	+	+	+	+	+	+	+	+	+
Casamino Acids	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	+	-	-	-	-	+	-
Formate (Formic Acid)	-	-	-	-	-	-	-	-	+	-
Fructose (D-)	-	+	+	-	+	-	+	+	-	-
Glucose (D-)	+	+	+	+	+	+	+	+	+	+
Glutamate (L-)	+	+	+	+	+	+	+	+	-	+
Lactose (α-)	-	-	-	-	-	-	-	-	+	-
Malate (L-)	-	-	-	-	-	-	-	-	-	-
Methanol	-	-	-	+	-	-	-	-	+	-
Pyruvate	+	+	+	+	+	+	+	+	+	+
Succinate	+	+	-	-	+	-	+	-	-	-
Yeast Extract	+	+	+	+	+	+	+	+	+	+
Antibiotic Sensitivity:										
Ampicillin	R	R	S	S	R	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S	S
Imipenim	R	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S
Penicillin G	R	R	S	S	R	S	S	S	R	S
Polymyxin B	S	S	S	S	S	S	S	S	S	S
Naladixic Acid	R	R	R	S	S	R	R	S	R	R
Streptomycin	R	R	R	R	R	R	R	R	R	R
Tetracyclin	S	S	S	S	S	S	S	S	S	S
Optimal growth:										
Vitamin Requirement	+	+	+	+	+	+	+	+	+	+
Temp Range °C	6-37	10-37	6-45	4-28	4-45	4-37	4-45	4-28	8-37	6-37
Optimal Temp °C	28	28	25	15	15	15	15	15	28	25
pH Range	5-10.5	5-10.5	6-10	5.5-10.5	6-10.5	6-10.5	6-10.5	6-10.5	5.5-10.5	6-10
Optimal pH	7	5	6	6	6	6	6	6	10	6
Growth at 0% NaCl	+	+	+	+	+	+	+	+	+	+
NaCl range	0 - <2%	0 - <2%	0 - <2%	0 - >2%	0 - >2%	0 - >2%	0 - >2%	0 - >2%	0 - >2%	0 - <2%
Anaerobic growth on RO, light (7 days)	-	-	-	-	-	-	-	-	-	-
Anaerobic growth on RO, dark (7 days)	-	-	-	-	-	-	-	-	-	-
Fatty acid composition (%):										
Primary	C18:1	C18:1	C18:1	C18:1	C18:1	C18:1	C18:1	C18:1	C18:1	C18:1
Secondary	C16:1	C16:0	C16:0	C16:0	C16:0	C16:0	C16:0	C16:0	C16:0	C16:0
Tertiary	C16:0	C14:0	C14:0	C14:0	C14:0	C14:0	C18:1n7c	C18:1n7c	C16:1	C14:0

R = resistant; S = sensitive; + = yes; - = no; Temp = Temperature.

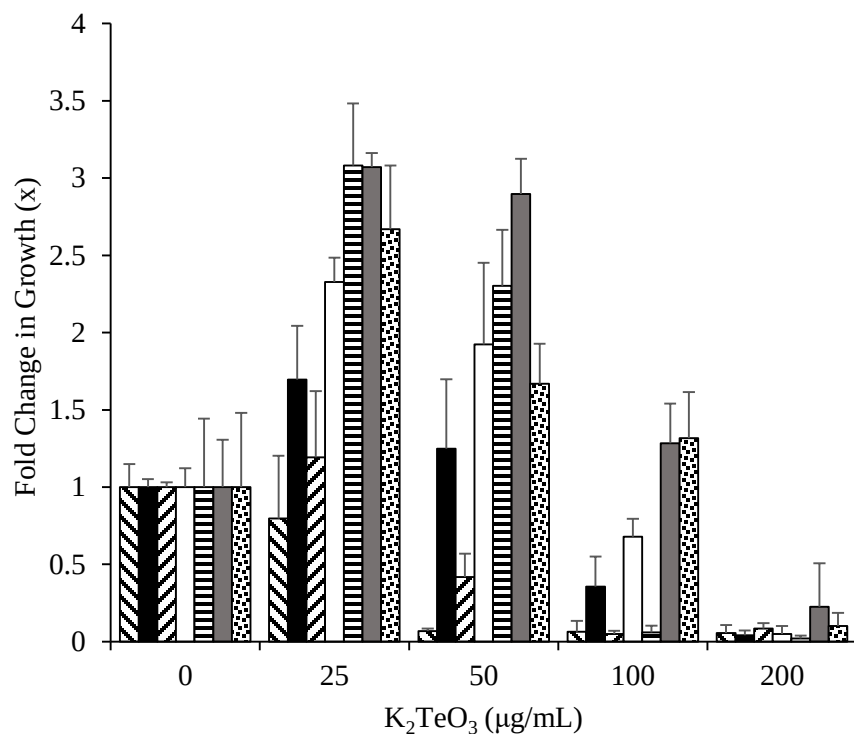


Fig. 3.11. Tellurite tolerance among zebra mussel AAP isolates. AAP strains Z1, Z2, Z7, Z24, Z39, Z59 and Z68 were cultivated in RO medium with increasing levels of K₂TeO₃, in corresponding order of bars left to right; downward stripe, black, upward stripe, while, horizontal stripe, grey, dots. After 7 days, growth with K₂TeO₃ was compared to cultures developed without and measured as a fold change.

microbial load. During sampling, few lone zebra mussels were washed upon the white sandy shore line at any beach in the fall of 2017. An extensive search had to be conducted to find mussels by venturing to scarce boulders and large stones. Upon examination of these large solid surfaces, it was clear that most had mussel beds already formed (Fig. 3.1C), confirming their foothold on the lake. Of note, numerous zebra mussels were evident during 2019 at the same sites, revealing their prolific reproduction during that short time. The lake's alkalinity (pH 8.9-9.0) was slightly higher than averages measured among the lake, with mean pH 8.2 (MARD, 2020). Analogous to other lakes and wetlands (Wilhelm *et al.*, 2020; Reid and Mosley, 2015), alkali pH during autumn has been attributed to changes in dissolved CO₂ content, either by its removal via active photosynthesis, or evapoconcentration, which can cause pH to rise due to the increase of alkaline solutes. Notably, increase in pH could be of positive influence to zebra mussels, as they prefer high pH, which prevents shell degradation from calcium loss (Claudi *et al.*, 2012).

3.6.2. Distribution of chemical elements among sites and zebra mussel samples

The elemental composition at each site were remarkably similar to one another, especially when considering the several kilometer distance between them (Fig. 3.1 and Table 3.1). Water measurements were the most stable, fluctuating less than in sediment. While summarized dissolved content of all 39 elements in water made up to 127.5, 137.6, and 161.9 ppm at S1, S2, and S3, respectively, sediment samples were comprised of 135,207.4, 26,483.0, and 7,990.7 ppm of total elements at the same sites. The similarity of water chemistry found in samples separated by 35 km was supported by the fact that Lake Winnipeg is a well mixed waterbody. The deviation in sediment could be predicted because of the variation in grain sizes of the sand collected, and the sieving technique would result in different overall concentrations. While this was the case, relative concentrations remained similar, and data proved that all important elements were still biologically available.

Entire zebra mussel homogenate analysis showed similar trends in elemental composition of bivalves collected from both beaches. In contrast to lake water, all elements were detected 10× higher in numbers within zebra mussels, except for Mg, which was only 6× more. Comparing zebra mussels to sediment, 7 elements are impressively elevated in bivalves, suggesting their significant bioaccumulation. Starting with the greatest ratio in *D. polymorpha*, Na was 17.5× of that in sediment. For a typical physiological ion in many eukaryotes, this was expected. Sr was 13.8× higher, and it has been documented to play a role in Ca uptake (Nielsen, 2004), assumedly important in shell formation. Ba was unusually high at 5.7×, and has been

previously found accumulated within diatoms (Thébault *et al.*, 2009), which indicated a possible consumption of diatoms by zebra mussels. Ca, while incredibly concentrated in zebra mussel homogenate at 174,500 ppm, was 4.7× higher than in the surrounding sediment. Such results are not surprising, as these organisms build their shells out of calcium carbonate. At 2.91× higher than in sediment, P was third most concentrated within mussels at 630.5 ppm, amassing this element within their tissues as was shown in the Great Lakes (Naddafi *et al.*, 2008). Next, incredibly high Cd at 2.39× of sediment, which has been linked to P levels in phytoplankton (Xu *et al.*, 2008), implicating a joint accumulation in bivalves. Cu, at 1.9× that in sediment, was accumulated in *D. polymorpha* similarly to several other bivalves (Martinci *et al.*, 1984; Chu *et al.*, 1990, Rzymiski *et al.*, 2014). While these studies found the accumulation of transition metals Cr, Co, Mg, Ni, Fe, Zn, Cd, Pb and Cu in mollusc tissues, our study discovered them to be elevated over 200× of that in the surrounding waters. Finally, Se was the last on the list of enriched elements within zebra mussels, at 1.13× higher than in sediment, and 584× of that in water. This metalloid has somewhat similar chemical properties with both Te and V, also these 3 are generally toxic to life in increased amounts. However, in moderate conditions it also has important positive biological functions and is included into the structure of some Se- containing enzymes (Köhrle *et al.*, 2000). While CRC ICPMS detection limits for Te were unfortunately not sensitive enough to determine its presence, V was 171× higher in mussels than in water.

3.6.3. Enumeration of bacteria on complex and selective media

Culture-based enumerations revealed similar numbers between all sites or tissues, where water and sediment counts were close to one another, as well as were those estimated in each respective bivalve tissue (Fig. 3.2). The similarities indicated that analogous bacterial communities existed on either side of the South Basin, and inside zebra mussels, as their functional capabilities in metabolism were comparable. Our observation is in good agreement with the idea that Lake Winnipeg's South Basin is well mixed, including both oxygen and nutrient availability (Kling *et al.*, 2011). In addition, each individual sample maintained comparable proportions of microbes capable of growth on each selective medium. This was surprising enough, as different ratios of aerobes to anaerobes could be expected when comparing habitats with potentially different oxygen content such as water and sediment, or the intestinal tract of an organism. However, in each case, regardless of sample type, microbes grown on RO and BG-11 were similarly prevalent, followed by 10-100× less microbes capable of either anaerobic growth on PNSM, or tellurite tolerance on RO_T. Taken together, bacterial populations had similar proportional numbers of heterotrophs, oligotrophs, and facultative anaerobes cultivated. Potentially, this may be due to the similarity of isolation approaches

and conditions for each sample. Since sediments were collected from the surface of submerged sand, which was well mixed in water, and zebra mussels are denoted for their high volume throughput in relation to size, both of these microcosms would not be drastically different from the surrounding waters. In all cases, pH, temperature, and oxygen availability were likely akin to each other. These parallel conditions would have caused similar functional groups of bacteria to proliferate at each site of isolation.

Since the highest counts were of aerobically grown heterotrophs, trends in numbers of cells cultivated on RO were specifically analyzed in detail and correlated between sample types and sites. In this way, total counts from water for all 3 sites were comparable, with 1.81×10^4 , 1.58×10^4 , and 2.08×10^4 CFU/mL for Gimli, Patricia, and Grand Beaches, respectively. Sediments at each site showed a little more variety, and the numbers were substantially higher than that observed in water, Gimli Beach with 5.35×10^6 CFU/g, Patricia Beach, 9.91×10^5 CFU/g, and Grand Beach the highest at 4.02×10^7 CFU/g. These results corroborated well with those observed microscopically from the Hudson River near zebra mussel beds (Frischer *et al.*, 2000), where water contained $10^4 \sim 10^5$ CFU/mL, and sediment had $10^7 \sim 10^8$ CFU/g. Our numbers may have been proportionally $10\times$ smaller than those found in the Hudson River due to existing differences between river and lake as habitats, including associated physical and chemical components, or from some variations in analytical approaches. While microscopy was useful to observe actual numbers of bacterial cells, culturing was informative to speculate about the metabolic potential of the population, although excluding some groups that did not grow on media used due to special requirements not provided in the artificially created medium. In comparison to the other two sites, water and sediment at Grand Beach maintained the highest counts of cells, which may have been the lack of influence by zebra mussels, which were absent during sampling, but could also be explained by habitat variation alone.

Within mussel tissues, similar trends for types of cultivated microorganisms existed between replicates and across sites (Table 3.3 and Fig. 3.2). Gills and gonads had 1.39×10^4 and 1.33×10^4 average CFU/g, respectively. These culturable microbial loads were quite reminiscent to one another, as well as to the counts from surrounding waters at either site. Conversely, gut counts averaged 3.03×10^5 CFU/g from both sites, which was higher by an order of magnitude than numbers established in their gill or gonadal tissues. In addition, the enumeration of bacteria within the gut were also up to 2 orders of magnitude lower than those found in sediment communities. In line with our findings, the Hudson River based study reported bacterial counts in whole mussel tissues to be higher than those in surrounding water, but lower than in sediments,

(Frischer *et al.*, 2000). Similar research from the Ohio River recorded zebra mussels gut tissues contained the highest numbers of bacteria, when compared whole tissue homogenate (Starliper *et al.*, 1997). At that time, 1.729×10^5 and 1.556×10^5 CFU/g were found in the gut, and combined tissue samples, respectively, when grown on a high-nutrient media, blood agar. Such results have supported our speculation, that similar microbial communities have likely been established within *D. polymorpha* at different locations.

Comparing all sites, AAP were cultured predominantly from environmental samples, with sediments containing the most, 2.17×10^6 CFU/g, at 13.98% of the heterotrophic population, followed by water with 3.17×10^2 CFU/mL representing 1.74% (Table 3.2). While not as high, animal tissues contained 6.64×10^1 , 3.48×10^3 , and 2.12×10^1 CFU/g in the gill, gut and gonad, respectively. These represented 0.48% of culturable heterotrophs on RO from homogenates of gills, 1.15% within their gut, and very few, around 0.16%, in gonadal tissues.

3.6.4. Tellurite tolerance detected among aerobic heterotrophs

Microbial tolerance to toxic levels of K_2TeO_3 among the culturable community was determined at each site by counting heterotrophs capable of surviving exposure to 100 μ g/mL since this level is viewed as a high level of resistance (Maltman and Yurkov, 2015). Analysis of samples from all three sites revealed that less tolerant organisms were present in water, 7.00×10^1 CFU/mL on average, when compared to the sediments at 2.79×10^4 CFU/g, and to those counted in zebra mussels, 3.21×10^2 , 2.75×10^3 , and 1.89×10^2 CFU/g in the gill, gut, and gonad tissues, respectively (Table 3.3). Here, we found that the microbes residing in sediments had the highest cultivated numbers capable of growing in the presence of the toxic oxide. Indeed, sediments had the highest abundance of most dissolved metals by several times (Table 3.1). Likely, any organisms in the proximity would have naturally evolved with a higher likelihood of tolerating metal oxides, specifically those that are toxic at higher concentrations, either accumulated historically within the sediment, or settled there due to runoff. Indeed, according to similar analysis in Lake Erie, bacterial communities adapted to the types and existing in the habitat levels of toxic heavy metals (Hoostal *et al.*, 2008). Relative to water, each investigated tissue example contained over 10 $\times$ more culturable bacteria capable of tolerating K_2TeO_3 . Moreover, their gut contained 100 $\times$ more tolerant bacteria grown at such conditions, which was in line with AAP counts throughout our samples. Such results indicated the possible bioaccumulation of Te- compounds by filter-feeders, as high levels of tolerance in bacteria are often present especially when there is a related selective pressure. Interestingly, 97% of CFU from all sources tested were coloured black after growth on

RO_T plates, while the medium itself remained translucent. The observed phenotype likely well correlated to internal Te- oxide reduction, rather than efflux, or some other known strategy discovered in a wide variety of bacteria, including AAP (Maltman and Yurkov, 2015; Presentato *et al.*, 2019), indicating that this reduction might be the primary resistance mechanism of the local community. While natural Te concentrations were unfortunately below equipment detection limits, several measurable metal(oid)s were accumulated within mussel tissues at elevated levels, including the elements Se and V, which share some properties with Te (Table 3.1). Supporting our results was the confirmation of Ti oxide accumulation by zebra mussels from the St. Lawrence River (Larios *et al.*, 2018), as well as the association of metal(oid) oxide reducing bacteria with other filter feeders (Maltman *et al.*, 2016), where they would transform toxic metal(oid) oxides to their elemental form within tissues of *Riftia pachyptila*, rendering them less harmful. Since bioaccumulation of metal(oids) occurs in *D. polymorpha*, most likely as oxides, it is reasonable that the mollusc associated microbiome has adapted to tolerate their heightened levels.

Interestingly, AAP were cultivated from RO_T at a greater frequency than RO without tellurite from all sites (Table 3.2). At 15.39% in water and 18.72% in sediment, AAP represented a reasonably high proportion of the cultured bacterial community capable of tolerating this toxic oxide. Within zebra mussels, few AAP were found in gill and gonadal tissues. However, of the total bacteria within the gut samples, 3.86% were AAP able to tolerate K₂TeO₃, suggesting metalloid oxide reduction may be most active in their digestive tract.

3.6.5. Genomic microbial community analysis of lake ecosystem and zebra mussels

Bacterial communities at each site and in tissue samples were visualized by separation at phylum level (Fig. 3.3). According to Faith's PD α -diversity analysis (Fig. 3.4), lake water and sediment had similar extracted bacterial genetic compositions. On average, the dominant DNA belonged to the phylum *Actinobacteria* estimated at 40%, followed by *Proteobacteria*, at 15%; 12% for *Bacteroidetes*, 11%; *Verrucomicrobia*; 9%, *Cyanobacteria*; 6.5%, *Chloroflexi*; 3.5%, *Planctomycetes*; 1.6%, *Acidobacteria*; and the remaining 1% was from DNA of *Armatimonadetes*, *Epsilonbacteraeota*, *Firmicutes*, *Gemmatimonadetes* and *Patescibacteria*. These proportions matched well with what has been observed in other numerous fresh water lakes (Newton *et al.*, 2011).

In combined zebra mussel tissues, our data revealed *Pseudomonas* as a dominant genus throughout, making up 22.2% among sequences observed. *Flavobacterium* was found next at 16.9%, followed by 14.9% for *Aeromonas*; 11.5%, *Acinetobacter*; 9.6%, *Arcobacter*; 5.4%, *Acetobacteroides*; 5.1%, *Deefgea*; 3.8%,

Bacteroides; and 2.9%, *Janthinobacterium*. These values corresponded well with previously published observations for Bivalvia (Kueh and Chan, 1985), and specifically, zebra mussels (Frisher *et al.*, 2000). This early study found *Pseudomonas* to be dominant too, while also detecting *Acinetobacter*, *Flavobacterium* and *Aeromonas* isolates. As for zebra mussels and their individual organs, our experimentation additionally disclosed γ -*Proteobacteria* primarily within tissues (Fig. 3.5), which was also reported in a small lake near Lake Michigan (Winters *et al.*, 2011). In addition, recent analysis of common North American freshwater bivalves has also shown mussel-specific microbiomes exist within natural environments, specific to each species of mussel (Weingarten *et al.*, 2019).

While water and sediment contained comparable bacterial communities between sites, zebra mussel tissue microbiomes were also similar to one another, where a divergence existed between *D. polymorpha* and their surrounding aquatic habitat (Fig. 3.4 and 3.7). Furthermore, gill, gut and gonad all had similar communities, visualized in the Jaccard Emperor β -diversity plot (Fig. 3.7). The gonad tissue contained the least diverse group of bacteria in samples from all sites (Fig. 3.4). In addition, the gut microbiome was statistically divergent from the gonad using Faith's Phylogenetic Diversity to differentiate bacterial communities (Table 3.4), also having a larger bacterial species diversity. Interestingly, gill samples shared representatives with both gut and gonadal tissues (Table 3.5). This commonality of community is understandable, as bivalve gills are located at the threshold of mantle cavity and intestinal tract, where only selected organisms would be able to pass into the gut, and all others would be collected outside the gill prior to pseudofeces secretion. Since our bivalve sampling anticipated microflora to be present from the inner and outer thresholds of each organ, data from tissues was contrasted with each other to denote deviation of species present. For this purpose, bacterial genes extracted from gut tissues were related to those coming from gonad as a ratio gut:gonad, and the genera more prevalent within gut flora were investigated. The highest representation within the gut was by the genus *Janthinobacterium*, at $249.2\times$ of gonadal numbers. Originally, it had been published as a genus associated with sea sponges (Gerwick and Sitachitta, 1999) and salamanders (Valdes *et al.*, 2015), and is a commensal bacterium that prevents pathogenic growth due to production of antimicrobials. Second highest was a *Flavobacterium sp.* that related to an isolate from *Hydra vulgaris* at $86.1\times$ of that in gonadal DNA. *Flavobacterium* have been associated to bivalve specific microbiomes (Kueh and Chan, 1985), whereas *Hydra* have been found associated with this bivalve (Zaiko *et al.*, 2009) as a commensal organism that prey on larger zooplankton (Stanković and Ternjej, 2010; Massimo, 2017). *Hydra* related *Flavobacterium sp.* prevalent within the stomach of *D. polymorpha*, suggested a

potential association with the polyp, which comes with a cost to the hydra by being consumed and digested. Third highest at 54× was *Peredibacter*, a known predator of *Pseudomonas* (Davidov and Jurkevitch, 2004). These two genera were inversely proportional in the gut and gonads, which indicated the predation premise of *Pseudomonas* by *Peredibacter*. Fourth, fifth, and sixth highest in the gut to gonad ratio at 44.8×, 29.4×, and 20.5×, were *Bacteroides*, *Proteocatella*, and *Candidatus Campbellbacteria*, each individually suspected for symbiosis in gastrointestinal tracts of marine life (Xu and Gordon, 2003; Pikuta *et al.*, 2009; Dudek *et al.*, 2017). Seventh in line at 19.3×, *Comamonadaceae*, with known AAP among them, may be associated as prey or symbionts with bivalves under study. The following *Flavobacterium*, *Acetobacteroides*, and *Bdellovibrio*, at 16.8×, 11.5×, and 10.7× that of the gonadal microbiome, have all additionally been suggested as gastrointestinal symbionts (Sicuro, 2015; Su *et al.*, 2014; Iebba *et al.*, 2014). *Bdellovibrio* is also a predator of Gram negative *Pseudomonas*, once again likely explaining the lower level (half as many) pseudomonads in the gut. Interestingly, the zebra mussel associated microbiome was distinct and divergent from the microbiomes of natral freshwater mussels observed in North American freshwater mollusks (Weingarten *et al.*, 2019), suggesting host specificity.

As an aside, the detection of *Patescibacteria*, including *Parcubacteria*, was notable since they are ultramicroorganisms with less than 0.3 µm diameter and very small genomes. While previously associated with environmental groundwaters (Tian *et al.*, 2020), their presence here suggests that they may be found in lake water/ sedimments in addition to ground waters, and may have a small role in these filter feeders as well.

Previous studies proved cyanobacterial and *E. coli* consumption by *D. polymorpha* (Vanderploeg *et al.*, 2001; Mezzanotte *et al.*, 2016). However, these microbes were all but absent in our zebra mussel samples. While cyanobacteria were observed in the ecosystem making up ~10% of 16S V4 rDNA sequences and ~10⁴ CFU/mL, *E. coli* was not detected, which was expected because it is typically a transient species in freshwater ecosystems such as Lake Winnipeg, coming from a temporary contamination of fecal origin. Thus, this snapshot of the zebra mussel microbiome may have under-represented the prey of these bivalves. Potentially, the combination of slow feeding rate during the cold sampling season (Costa *et al.*, 2008), and quick digestion of bacteria once in the gut may be the cause. Since they have a rapid filtration rate, active consumption must involve swift digestion of nutrients prior to ejection of undesired effluent. In addition, the prevalence of

several genera of bacterial predators within their gut may have aided in the rapid consumption of other microbes, supporting our speculation.

As mentioned in Section 3.5.2, the proportions of AAP among the cultured heterotrophic populations were 0.48, 1.15, and 0.16% in the gill, gut and gonad tissues, respectively. Cellular estimates of AAP were remarkably similar to those obtained via the sequencing of extracted community rDNA. Here, we found 0.22, 1.13, and 0.20% of the 16S V4 rRNA genes in the gill, gut, and gonad belonged to AAP. The comparable results achieved via culture and sequence based methods put forward the idea that almost equal proportions of heterotrophic populations existed between communities found in both analyses. The correlation of phenotype to genotype was obvious, as a majority of bacteria found in our sequences (predominantly pseudomonads) were described as aerobes that can be successfully cultivated in RO medium. In agreement, zebra mussel organs have plenty of nutrient availability (Table 3.6), as their tissues and digested nutrients within the gut are excellent organic sources for microbes. While these cross-method based results were comparable for mussel associated microbes, the data for water communities revealed less resemblance, with even fewer similarities for the sediment microbial consortium. While 1.74% of cultured heterotrophic population in water were AAP, they made up to 2.78% of 16S V4 rRNA sequences. Conversely, in sediment samples 13.98% of the cultured heterotrophs were AAP, however only 0.88% of the 16S V4 rDNA sequences originated from photoheterotrophs. These differences were likely related to the expanded diversity of species observed in sequences from both water and sediment, of which many species are likely not capable of growth on the provided media or at cultivation conditions. It has been well advertised that most bacteria existing in nature are not yet culturable due to unknown requirements, and more than 99.999% have yet to be cultivated (Locey and Lennon, 2016). Hence, extraction from habitats and sequencing the bacterial genes may provide some information on phylogenetic diversity, but much needs to be done via cultivation to discover the metabolic richness. Despite these limitations, our results still consistently confirmed that AAP are present across the eutrophic waters of Lake Winnipeg. While the average cultivated AAP in water was detected at 3.17×10^2 CFU/mL during the fall sampling, the numbers may be lower than actually present in the lake, due to some possible limitations of used cultivation techniques. Microscopic analysis of a small Lake Vilkov found that AAP make up $\sim 5 \times 10^5$ cells/mL in the fall (Kolářová *et al.*, 2019). However, while also freshwater, this European small lake was recently man-made, and was only covered with ice for less than 2 months per year, therefore results received there should be used for comparison with some reservations. Nevertheless, if microscopic analysis would indeed render a higher population of AAP in Lake Winnipeg,

then in the sediments, which found to contain 2.17×10^6 CFU/g, then it is indeed an environment worth future exploration with microscopic methodology. Furthermore, as our samples were taken during the fall season, future attempts may reveal greater numbers, as it has been suggested that the AAP population may increase during warmer months (Koblížek, 2015).

3.6.7. Diversity of isolated AAP

After enumeration, pigmented isolates were tested to identify representatives that contained BChl a and carotenoids (Tables 3.2 and 3.5). Aerobic BChl a production was confirmed by the presence of absorption peaks at 770 nm, measured in acetone-methanol cell extracts (Table 3.5). In addition to BChl a, all AAP collected from zebra mussels contained carotenoids and were orange or red-orange coloured. Near complete sequences of 16S rDNA related all strains to the family *Sphingomonadaceae*, in particular to the genera *Porphyrobacter* and *Erythromicrobium* (Fig. 3.8). While this family was detected in our bivalve tissue genomic community analysis, there were no representatives from other families containing AAP, like *Comamonadaceae*. The absence may be explained by their specific growth factor requirements to trigger BChl a synthesis, similarly to published *Roseateles depolymerans*, an AAP within the family that needed enrichment with poly-hexamethylene carbonate to yield measurable amounts of BChl a (Suyama *et al.*, 1999). In our experiments, some AAP colonies could have been cultivated on plates, but possibly failed to synthesize detectable amounts of BChl a, because of a lack of some required stimulating factors (Yurkov and Beatty, 1998). Use of the species genomic information presented in our work may advance research towards successful enrichments for such demanding organisms in the future.

While many purified AAP isolates from mussels had high 16S rRNA sequence similarity to known type species (Table 3.5), Z24 and Z39 only resembled type species *Porphyrobacter sanguineus* by 98.7 and 98.9%, respectively. The lower homology of these two strains may speak about their taxonomic status as new species within this genus, as distance became even more apparent once a specific maximum likelihood tree was established with 10,000 bootstrap iterations (Fig. 3.9). Further confirmations are needed to address the speciation among *Erythrobacteraceae*. Comparison of physiological traits found that the cellular motility of each isolate matched fairly well with phylogenetic relatives. Strains Z24 and Z39, genomically similar to *P. sanguineus*, as well as Z6, Z59, and Z68, related to *Porphyrobacter neustonensis*, were all motile during exponential growth phase (Table 3.6 and Fig. 3.9). In addition to usual AAP identification features, such as aerobic carotenoid and BChl a production and sequence similarity, strains were found to have some

interesting phenotypic peculiarities (Table 3.6), including that all strains did not require NaCl, which is coherent with the freshwater origin of their habitat. However, closely related type species *P. sanguineus* required 1% NaCl for optimal growth (Hiraishi *et al.*, 2002), supporting further differentiation of our isolates. All strains could not grow anaerobically, strongly confirming that physiologically they were AAP. Optimal growth temperatures ranged from 15-28 °C, many of which were lower than that of related strains that grow optimally at 30-48 °C. This feature may be an adaptation to conditions associated with the cold humid continental climate zone of the place of isolation. Furthermore, while our strains were ovoid or short rods when grown exponentially, an elongated cellular morphology appeared after 3 days during stationary phase (Fig. 3.10). Such changes reflected different morphological life periods, with motility occurring while nutrients were plenty, followed by a filiform change when nutrients were exhausted. This premise coordinated well with their phototrophy strategy, as more pigmentation was also observed in the late growth phase (Yurkov and Beatty, 1998). Formation of long undivided cellular structures may allow for the creation of a matrix with filamentous cyanobacteria in mat-like structures, where light, as well as nutrients shed from primary producers, are available.

3.6.8. AAP growth stimulation and inhibition by metalloid oxide K_2TeO_3

Most AAP have survived high levels of extremely toxic metalloid oxides when challenged (Maltman and Yurkov, 2015), therefore we tested new isolates for growth in liquid RO with increasing concentrations of K_2TeO_3 until complete inhibition (Fig. 3.11). As expected, representatives within each morphogroup (Fig. 3.9) behaved similarly, and differences existed between strains expressing individual phenotype or genotype. Every investigated AAP strain survived at least 25 µg/mL, and was inhibited by 200 µg/mL. Obviously, they were not as adapted to extremely high levels of tellurite as previously published AAP, but were still substantially tolerant, as 1 µg/mL is already extremely toxic to most microorganisms (Yurkov *et al.*, 1996). Of course, MICs were important factors, but the growth enhancement for most strains under K_2TeO_3 sub-inhibitory levels was truly interesting. A majority of strains had a higher final optical density in the range 25 to 50 µg/mL, which may have indicated a growth promotion from the reduction of metalloid oxides similarly reported for other organisms (Maltman *et al.*, 2016). Indeed, inner cellular reduction of TeO_3^{2-} to elemental Te may be a common phenotype of AAP. However, this feature cannot be used for fast AAP identification, since many other strict heterotrophs and some photoheterotrophs could perform the same reactions at such relatively low concentrations of TeO_3^{2-} .

3.6.9. AAP association with crustaceans and possible metalloid oxide correlation

When considering the niche of AAP in aquatic environments, few studies tentatively spoke about their predation by higher forms of organisms. While recent work considered a possibility of their predation by zooplankton (Ferrera *et al.*, 2017), previously isolated AAP among hot springs were described as associated with *mollusca* (Bilyj *et al.*, 2014), in the vicinity of black smoker plumes with tube worms (Yurkov *et al.*, 1999; Maltman *et al.*, 2016), and in the gut of sea cucumbers (Jung *et al.*, 2014). Other strains, all identified based solely on 16S rRNA sequence data as members of the genus *Roseobacter*, have been studied for their ability to produce antibacterial agents (Ruiz-Ponte *et al.*, 1999), for symbiotic relationship with cuttlefish (Grigioni *et al.*, 2000), and for the potential to cause juvenile oyster disease (Boettcher *et al.*, 2000).

In our study, AAP were predominantly cultivated from the gut of *D. polymorpha*, making 1.15% of total bacteria, and showed even higher presence in Lake Winnipeg's littoral zones, 1.74% in water and 13.98% in sediment. Culture-based results were supported by similar proportions established from 16S V4 rRNA from eDNA sequencing based methods. Considering facts above, predation of AAP by mussels is therefore proposed. The capability to form chain-like morphology observed in our isolates, made these otherwise small bacteria, more susceptible to predation. In addition, AAP attach to suspended particles in open water (Waidner and Kirchman, 2007), which would also increase their overall mass, and indirectly raise the likelihood to meet the size requirement of filtration feeders.

In summary, we found zebra mussels bioaccumulated many elements, including Se and V. Since both are somewhat similar in properties to Te, and all isolates were significantly tolerant to K_2TeO_3 , it was logically likely that *D. polymorpha* also accumulated somewhat elevated concentrations of Te- oxides and the bacterial community associated with mussels were capable of surviving its high levels. Under such circumstances, a symbiotic relation may exist, where AAP are performing metalloid oxide reductions, rendering them less toxic to the coexisting organisms, including *D. polymorpha*, analogically to recently discovered association of metalloid oxide reducers with tube worms in the vicinity of "black smokers" (Maltman *et al.*, 2016).

As these bivalves have been shown to select against species of cyanobacteria (Vanderploeg *et al.*, 2001), very likely they could also select for other specific groups of bacteria. Since experimentally, zebra mussels fed on clinical bacteria given no alternative (Mezzanotte *et al.*, 2016), or in contaminating effluent (Bighiu *et al.*, 2019), future work should specifically monitor organisms, including AAP naturally inhabiting lake ecosystems, to identify the preferred prey. Considering the invasive nature of *D. polymorpha*, methods to

slow their proliferation may be developed by either recognition of bacteria consumed, or determining their local flora. Any cohort of associated bacteria could potentially be targets of elimination to reduce their digestive capability, and therefore impede the animal's growth.

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4. Chapter 4.

Physiology and phylogeny of 20 aerobic anoxygenic phototrophic species isolated from a single freshwater habitat provide insight into an ecological niche

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4.0. Preamble

To assess the population of aerobic anoxygenic phototrophs inhabiting the 11th largest freshwater lake in the world, Lake Winnipeg, seasonal sampling over 5 years was carried out at littoral and limnetic sites to collect water and sediment for their enrichment. Over 500 isolates were purified using a variety of growth media, and 150 were phylogenetically identified via 16S rRNA gene sequencing due to perceived unique characteristics. These strains collectively related to 20 different type species.

All hailed from α -*Proteobacteria*, where 6 were within *Sphingomonadaceae*; 7, *Erythromonadaceae*; 1, *Sphingosinicellaceae*; 4, *Methylobacteriaceae*; 3, *Acetobacteraceae*; and 1, *Rhodobacteraceae*.

Multiple physiological features were analyzed from representatives of all 20 species. We found overwhelming evidence of familial homogeneity, while also revealing the diversity among families that may help to explain a specific ecological niche for each taxonomic group.

4.1. Contributions statement

Conceptualization, S.B.K. and V.Y.; methodology, S.B.K. and V.Y.; software, S.B.K.; validation, S.B.K., V.Y.; formal analysis, S.B.K., K.M. and J.P.; investigation, S.B.K., K.M., J.P. and K.W.; resources, V.Y.; data curation, S.B.K.; writing—original draft preparation, S.B.K.; writing—review and editing, S.B.K., V.Y.; visualization, S.B.K. and V.Y.; supervision, V.Y.; project administration, V.Y.; funding acquisition, V.Y.

While not yet published, this study is the culmination of all bacterial physiological work completed during S.B.K.'s PhD program. S.B.K., X.M. and K.W. were involved in purifying the original 20 strains described, while S.B.K. and V.Y. made the decisions on which to analyze in details. S.B.K. and J.C. initiated experiments on metal(loid) tolerance of 7 strains, whereas S.B.K. and K.M. completed this work with the remainder of the isolates. S.B.K., X.M. and K.W. started physiological testing with 10 AAP, and S.B.K. and K.M. finalized the rest. S.B.K. completed all DNA work, microscopy, qualitative and quantitative analysis to make final conclusions with V.Y. and wrote the article/ chapter.

4.2. Abstract

While investigating aerobic anoxygenic phototrophs (AAP) from Lake Winnipeg's bacterial community, over 500 isolates were obtained. Relatives of 20 different species were examined simultaneously, identifying conditions for optimal growth or pigment production. All were phylogenetically distributed among α -Proteobacterial families *Erythrobacteraceae*, *Sphingomonadaceae*, *Sphingosinellaceae*, *Acetobacteraceae*, *Methylobacteriaceae*, and *Rhodobacteraceae*. Phenotypic characteristics matched genetic relationships, where morphology, physiology, pigmentation, metal transformations, lipid configurations, and enzymes were comparable within, but distinctly separated between families. Bacteriochlorophyll synthesis was promoted under oligotrophy for all strains, whereas copiotrophy repressed production. New AAP diversity was also reported by revealing strains related to non-phototrophic *Rubellimicrobium*, and to genera with only few previously identified AAP species, including *Roseomonas*, *Sphingomonas*, and *Methylobacterium*/*Methylorubrum*. Detected photosynthesis exposed these genera to predominantly contain AAP, rather than previous conjecture of strict heterotrophic members. *Sphingomonadales* strains contained the highest tellurite tolerance, 2000 $\mu\text{g/mL}$, matching previous findings, whereas *Methylobacteriaceae* and *Acetobacteraceae* had selenite tolerance up to 1000 $\mu\text{g/mL}$, revealing widespread metal transformations that are likely an important ecological feature. This study exemplified the overwhelming diversity of AAP in a single aquatic environment, confirming cultivation continues to be of importance in microbial ecology to discover functionality in both new and previously reported cohorts of bacteria.

Keywords: Aerobic anoxygenic phototrophs; Bacterial taxonomy; Physiology and phylogeny of phototrophs; Bacteriochlorophyll and carotenoid production; Microbial ecology

4.3. Introduction

Aerobic anoxygenic phototrophs (AAP) are bacteria that use bacteriochlorophyll (BChl) containing reaction centers (RC) and light harvesting (LH) complexes in the presence of oxygen to generate biological energy via photosynthesis (Yurkov and Beatty 1998, Yurkov and Hughes, 2017). While all known species lack significant autotrophic pathways to fix CO₂ and create organics from inorganics (Hauruseu and Koblížek, 2012; Yurkov and Hughes, 2013), they have been ubiquitous in aquatic ecosystems, representing a substantial part of microbial communities of euphotic zones (Koblížek, 2015). Recently, Lake Winnipeg, the 11th largest freshwater lake in the world was investigated for its bacterial composition, with a major focus on phototrophic populations (Chapter 2). In that work, AAP were found prevalent in the spring season, up to 1.49 and 1.02% of the sequenced community, and 19.1 and 4.7% of the laboratory-culturable copiotrophic heterotrophs, at littoral and limnetic zones, respectively. Decreasing throughout the summer, they followed the amount of available sunlight each year (Chapter 2). Although AAP have been quantified in other aquatic systems with valuable culture-independent methods including genetic analysis (Piwosz *et al.*, 2022) or infrared autofluorescence microscopy (Mašín *et al.*, 2008; Čuperová *et al.*, 2013), such detection techniques have had limited success in describing functional physiological features of any predominant population.

The isolation and characterization of unique strains has thus remained important, since the ecological role of AAP and their fit into aquatic foodwebs and biogeochemical cycling is still ambiguous. As photoheterotrophs, they require the presence of organic building blocks for assimilation, yet ATP can be synthesized via either photophosphorylation or predominantly oxidative phosphorylation (Yurkov and van Gemerden, 1993; Hauruseu and Koblížek, 2012). Photosynthesis has been further shown to occur on a diurnal cycle in situ (Fecskeová *et al.*, 2019), and they are capable of consuming various organic molecules resulting in net CO₂ emission from respiration (Piwosz *et al.*, 2022), but the balance between these two energy production pathways in natural settings has yet to be resolved. In addition, most taxonomically identified AAP have come from vastly different ecosystems (Yurkov and Hughes, 2017). Here, we purposely and intentionally studied many AAP strains from a common singular habitat, that each genetically related to either known or unknown phototrophic species. This complex side-by-side comparison provided clear grouping of characteristics that were not otherwise blurred by contrasting results to isolates accustomed to different habitats or growth conditions.

In this way, the diversity of primary and secondary pigment production and the comparison of physiological traits of multiple distinct AAP populations from a single habitat led to the recognition of features that unified them, while also distinguishing between individual groups. This in turn, permitted a well-supported speculation about their ecological functions in nature.

4.4. Material and Methods

4.4.1. Specimen collection

Bacterial strains listed in Table 4.1 were isolated from freshwater Lake Winnipeg samples collected spatially and seasonally; specific site locations and media used have been previously described (Chapter 2). Following purification, all strains were cryopreserved, or maintained on RO medium at 28°C in the dark during analysis. Briefly, a thick cell suspension was frozen at -75 °C in the above-mentioned media supplemented with 30% glycerol as a cryoprotectant. Growth experiments used 5% inoculum in 8 mL medium with 6" test tubes rotor-aerated for 7 days in triplicate to reach stationary phase, unless otherwise noted.

4.4.2. Pigment extractions and absorption spectra

Spectra were recorded from strains grown under aerobic conditions on agarized plates as pigmentation was visually deeper than in liquid cultures. Whole cell samples were prepared by resuspending in 0.3 ml 20 mM pH 7.8 Tris/HCl buffer and 0.7 mL glycerol to reduce light-scattering (Rathgeber *et al.*, 2005). Pigments were extracted by collecting cells via centrifugation from 1 mL culture, removing supernatant, then adding 0.5 mL 7:2 acetone:methanol. Cellular debris was later pelleted to not interfere with readings. Intact bacteria and extract absorption spectra were then recorded using a Hitachi U-2010 spectrophotometer between 300 and 1100 nm with a 10 mm path-length quartz cuvette. Samples were compared to standards for the confirmation of LH and RC complexes, extracted carotenoids and BChl, and to denote unusual maxima characteristics of new strains (Yurkov and Beatty, 1998). Concentrations of bulk carotenoids and BChl a were individually approximated in extracts from absorbance peaks at 450 and 770 nm, respectively.

4.4.3. Physiology and morphology

The utilization of single organic substrates; hydrolysis of Tweens, gelatin, starch, agar; catalase, oxidase, indole, or nitrate reduction enzyme activities; antibiotic sensitivities, fermentation and utilization of simple sugars were determined as described for several AAP type species (Yurkov and Van Gernerden, 1993; Yurkov *et al.*, 1994). Anaerobic photoheterotrophic growth was tested under illuminated conditions in filled screw-capped tubes containing RO, PNSM, or alternative electron donor's indicative of anaerobic anoxygenic

photosynthesis including sulfide, thiosulfate, and reduced malate. Photoautotrophic CO₂ fixation was similarly attempted in light with oxygen present in organic-free medium (Yurkov and Beatty, 1998). Lipids were extracted in triplicate, using a chloroform: methanol methylation method (Bligh and Dyer, 1959), and analyzed by gas chromatography to determine fatty acid composition. Cellular size, shape and motility of exponential or stationary-phase cultures were determined using a phase-contrast light microscope (Zeiss Axioskop 2).

4.4.4. Growth experiments

To determine both optimal conditions and the range of growth capabilities, each strain was first cultivated in diapason of various temperatures from 4 to 44°C with 4°C steps. Growth at differing pH was also analyzed by modifying medium to pH 4.0 - 13.0 (0.5 intervals) by additions of HCl or NaOH to sterilized media prior to inoculation. Sensitivity to limiting or excessive available organics was studied using RO prepared without complex nutrient source “yeast extract”, and instead supplemented at 0, 0.1, 1, 5, 10, 25, 50, 150, 250, or 350 g/L in triplicate individual tubes. Tellurite and selenite tolerances were examined by adding 0, 25, 50, 75, 100, 175, 250, 500, 750, 1000 or 1500 µg/L of either metalloid oxide in RO to determine level of tolerance and oxide reduction (Maltman and Yurkov, 2015). All growth range experiments had total protein measured after the 7-day incubation. Briefly, cells from 1 mL of liquid culture were centrifuge pelleted and washed with 20mM Tris-HCl, pH 7.8 buffer prior to a second pelleting and suspension in 1 mL of some buffer with 1 mL of 1N NaOH that was boiled for 20 min to hydrolyze proteins. These were then quantified by a BCA assay kit (Sigma-Aldrich, USA). A 1 mL aliquot of culture was also collected to extract pigments from each condition as described above.

4.4.5. DNA preparation and phylogeny

Genomic DNA was extracted and 16S rRNA gene sequences were determined via Sanger sequencing using universal primers, and chromatograms were compiled into ~1450 bp fragments with DNA Baser Assembler v4.36.0 (Heracle BioSoft SRL, Romania). Sequences were aligned and compared to DNA of type species available from the NCBI nucleotide sequence database (Coordinators, 2013). Phylogenetic trees were built by Mega X software, neighbor joining alignment, Maximum Likelihood, and bootstrapping methodology at 1000 replicates (Kumar *et al.*, 2018). While a smaller tree was constructed using a Tamura-Nei model (Tamura and Nei, 1993) with the highest log likelihood of -10817.29 and 44 sequences of 1642 bp positions, a more comprehensive evolutionary tree was created from the General Time Reversible model (Nei and Kumar, 2000) and the highest log likelihood of -24976.44 from 123 sequences and a total of 1709

bp positions. The 16S rRNA gene sequences from strains AJ72, AM19, AM27, AM91, BA23, BC100, BE100, BK61, BL67, CK155, CK182, CL63, CN8, and GM14 were obtained from Genbank under accession numbers MW970361.1-MW970374.1, respectively. New sequences of CO70, BJ40, BL27, BL40, AE24, CB142, CE55, CI117, and AC101 were deposited to GenBank as OP491624.1-OP491632.1, respectively.

4.5. Results

4.5.1. Identification of new AAP isolates

Investigating Lake Winnipeg's microbial communities, >500 AAP isolates were collected and purified from various media (Chapter 2) and later cryopreserved, Section 4.4.1. Final strain selection involved both spectrophotometric and genetic considerations to narrow the focus on representatives with distinct characteristics. Over 150 AAP with atypical spectra and cell shapes had 16S rRNA genes sequenced, relating them to 20 known individual type species. Representatives of each taxo-group were then selected for further examination (Table 4.1).

4.5.2. Pigment analysis

A range of interesting whole-cell RC and LH absorption maxima was discovered from the chosen isolates, with peaks indicative of LHI blue-shifted down to 854 nm found for CK155, and red-shifted up to 875 nm in BC100 and CL63 (Table 4.1; Fig. 4.1). In comparison, LHII complexes absorbing at 834-836 nm were detected for AM27, AM19, and BL27, whereas BL67 had an unusual for AAP putative LHII at 855 nm. All isolates contained BChl *a* as their primary pigment for anoxygenic photosynthesis (Fig. 4.2). Various colorations were observed, with carotenoid spectra grouping by colour and major peaks. Those that had predominant orange hue included AM27, CN8, AM91, BL27, CK182, AM19, BE100 with 458 and 478 nm peaks, while brown to yellow BL67, BL40, BC100, GM14, BJ40, BK61, CO70 and AJ72 had 454 and 481 nm maxima. CB142, CL63, BA23 and CE55 were bright pink with 361, 497, and 527 nm, whereas AC101 was fuchsia pink, CK155, CI117, and AE24 were pastel pink, but all four had had predominantly 496 and 525 nm peaks (Fig. 4.2).

4.5.3. Microscopy, physiology, and phenotypes

Morphological diversity included several standard rod-like, ovoid and coccoidal strains, in addition to less common appearances of coccoid pairing in AE24, branching of CO70, or pleomorphic BK61 (Fig. 4.3). In addition to the classic bacterial shapes, we observed 2 distinct novel cellular arrangements. Strains CL63, BA23, and CE55 were all vibrioid, but far from typical slender "bent-rods", instead averaging 1.6 wide by 4.0 μm long (Fig. 4.3). To describe this, as no bacterial morphological term has been established for such cellular

Table 4.1. AAP isolate identifying characteristics. Isolate source, identity and important absorbance maxima observed in whole cells of each strain and related type species.

Year	Season	Site	Type	Medium	Strain name	Carotenoids and cytochromes	RC (nm)	LHII (nm)	LHI (nm)	Highest 16S rRNA sequence similarity	%
2017	Summer	4	liquid	OM	BL40	432,457, 488, 590	798	-	868	<i>Porphyrobacter colymbi</i>	99.43
2017	Summer	10	liquid	Potato	BE100	431, 462, 492, 592	801	-	868	<i>Porphyrobacter colymbi</i>	99.28
2017	Summer	4	liquid	RO	BL27	464, 494, 590	798	835	867	<i>Porphyrobacter tepidarius</i>	99.14
2017	Fall	5	liquid	RO	CN8	461, 494, 592	802	-	869	<i>Porphyrobacter neustonensis</i>	97.8
2017	Spring	4	sediment	OM	AM91	460, 492, 597	800	-	868	<i>Porphyrobacter sanguineus</i>	98.51
2017	Fall	3	sediment	TM	CK182	413, 460, 490, 536, 591, 640	802	-	867	<i>Erythromicrobium ramosum</i>	99.55
2017	Spring	4	sediment	TM	AM19	432, 460, 490, 590	798	836	867	<i>Erythromicrobium ramosum</i>	99.71
2017	Spring	4	sediment	TM	AM27	419, 459, 490, 541, 595, 643	798	834	868	<i>Erythromicrobium ramosum</i>	99.5
Related Freshwater Type species					E5	432, 458, 487	797	833	867	<i>Erythromicrobium ramosum</i>	N/A
2017	Spring	3	liquid	TM	AJ72	430, 460, 490, 595	797	-	863	<i>Sphingorhabdus lacus</i>	99.65
2017	Summer	3	sediment	OM	BK61	432, 458, 490, 591	802	-	867	<i>Blastomonas fulva</i>	99.08
2017	Summer	3	liquid	OM	BJ40	455, 488, 587	800	-	869	<i>Blastomonas natatoria</i>	99.86
2016	Fall	3	sediment	RO	GM14	432, 461, 492, 590	800	-	870	<i>Blastomonas ursincola</i>	99.3
Related Freshwater Type species					KR99	455, 488, 587	802	-	869	<i>Blastomonas ursincola</i>	N/A
2017	Fall	5	sediment	RO	CO70	432, 461, 492, 593	802	-	863	<i>Sphingomonas fonticola</i>	99.6
2017	Summer	8	liquid	TM	BC100	432, 459, 494	799	-	875	<i>Sphingomonas yangtingensis</i>	96.61
2017	Summer	4	liquid	Potato	BL67	433, 461, 490, 542, 591	800	855	869	<i>Sandarakinorhabdus cyanobacteriorum</i>	98.93
2019	Fall	7	liquid	Potato	CB142	477, 508, 548	798	-	869	<i>Methylobacterium hispanicum</i>	99.01
2017	Fall	4	liquid	OM	CL63	411, 481, 506, 548	798	-	869	<i>Methylorubrum extorquens</i>	99
2017	Summer	6	liquid	RO	BA23	412, 478, 503, 541	800	-	869	<i>Methylobacterium indicum</i>	96.18
2019	Fall	10	sediment	OM	CE55	475, 509, 544	798	-	869	<i>Methylobacterium terrae</i>	97.11
Related Freshwater Type species					RB3	472, 508, 540	799	-	856	<i>Roseococcus thiosulfatophilus</i>	N/A
2017	Fall	3	sediment	Potato	CK155	471, 503, 542	798	-	854	<i>Roseomonas sediminicola</i>	98.1
2019	Fall	2	sediment	R2A	CI117	472, 501, 541	798	-	859	<i>Roseomonas lacus</i>	98.55
2017	Spring	10	liquid	OM	AE24	470, 503, 542	798	-	853	<i>Roseomonas stagni</i>	98.83
2019	Spring	8	liquid	OM	AC101	471, 502, 540	798	-	863	<i>Rubellimicrobium rubrum</i>	96.16

Putative RC, reaction center; LH, light harvesting complex; -, not detected.

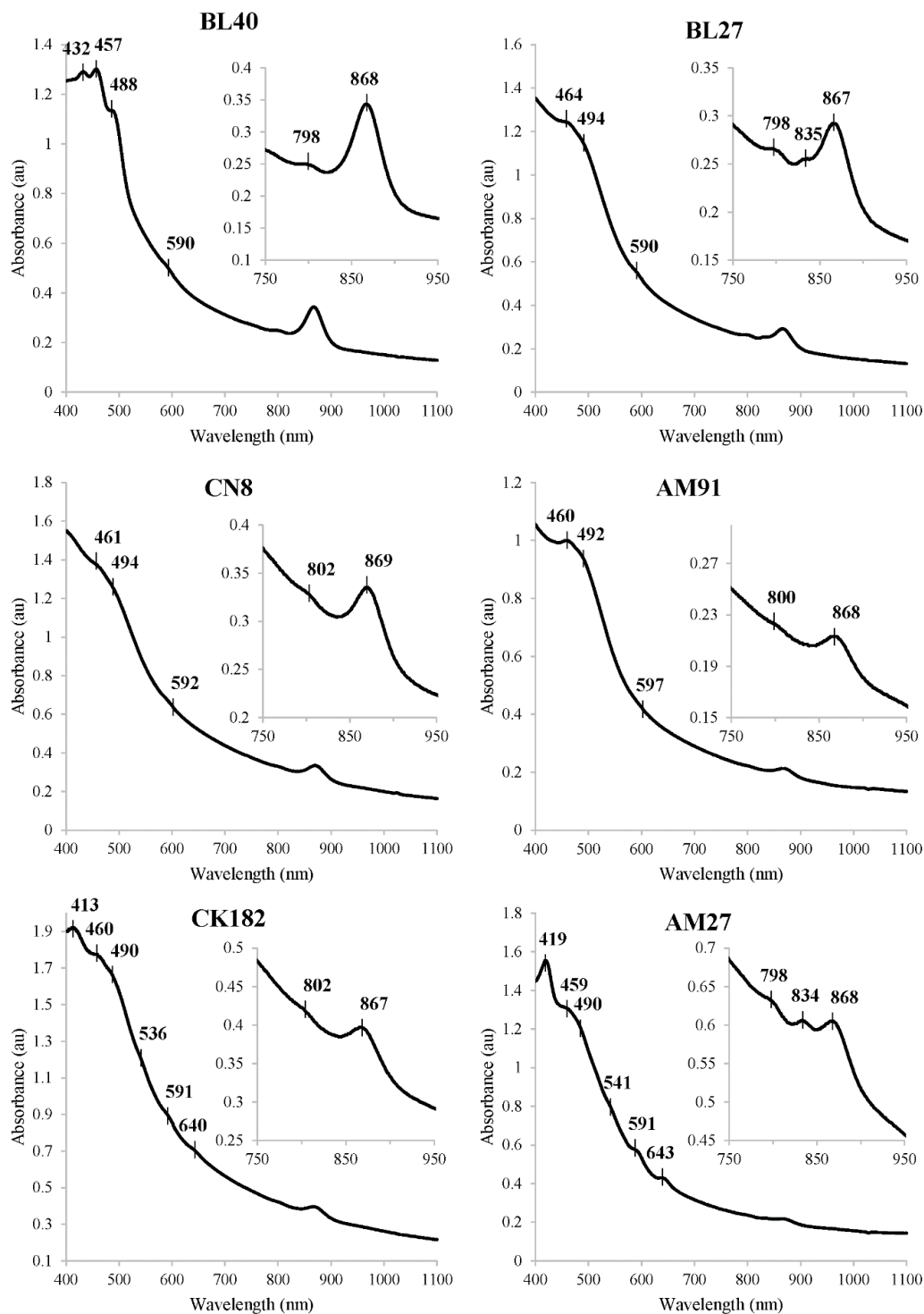


Fig. 4.1A. Whole cell spectra of each isolate. (A) *Erythrobacteraceae*, (B) *Sphingomonadaceae* and *Sphingosinellaceae*, (C) *Methylobacteriaceae*, (D) *Rhodobacteraceae* and *Acetobacteraceae*, (E) Extra strains and type species absorbance trace from 380-1100 nm, with major peaks and shoulders of photosynthetic pigments marked.

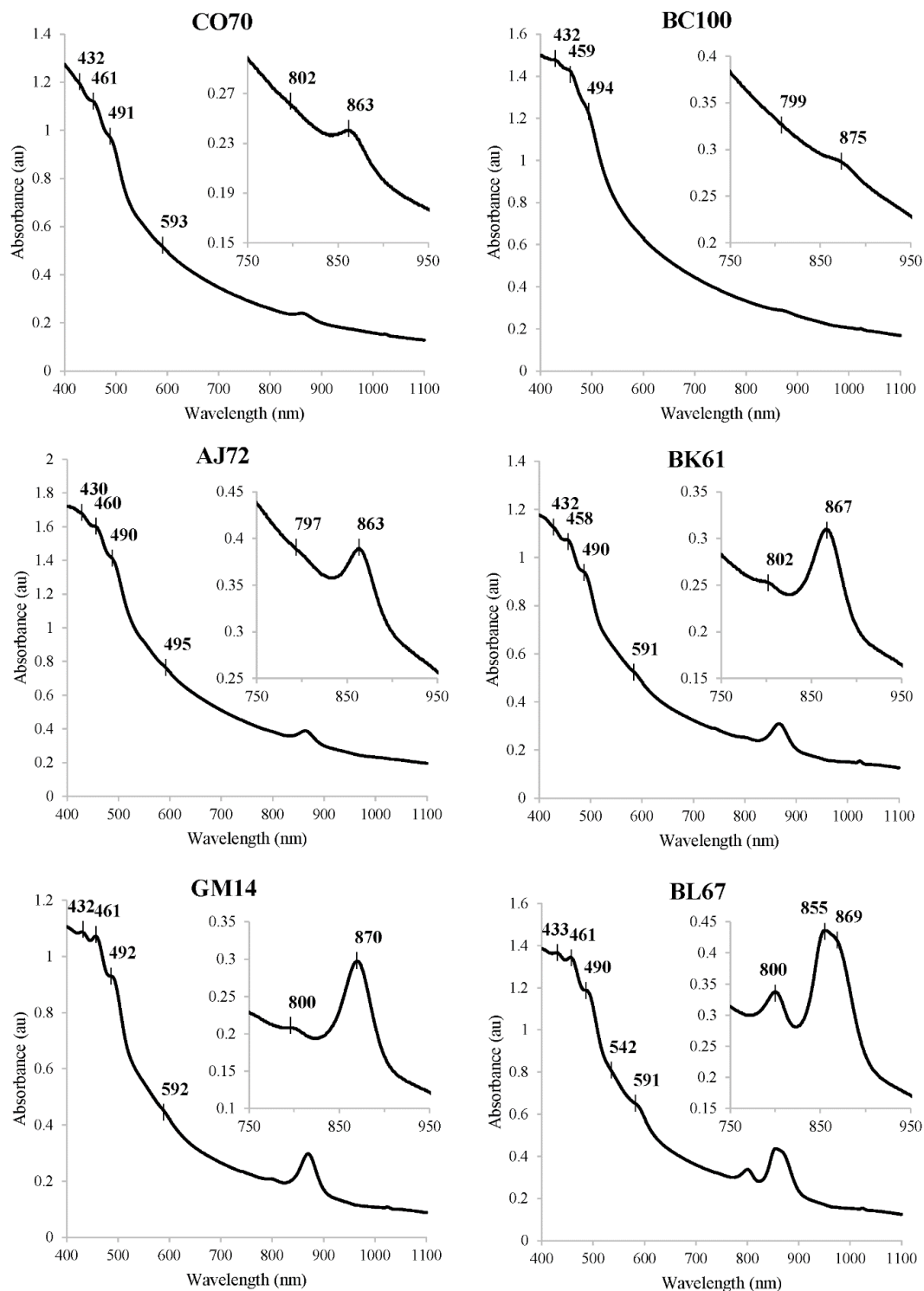


Fig. 4.1B. Whole cell spectra of each isolate. (A) *Erythrobacteraceae*, (B) *Sphingomonadaceae* and *Sphingosinicellaceae*, (C) *Methylobacteriaceae*, (D) *Rhodobacteraceae* and *Acetobacteraceae*, (E) Extra strains and type species absorbance trace from 380-1100 nm, with major peaks and shoulders of photosynthetic pigments marked.

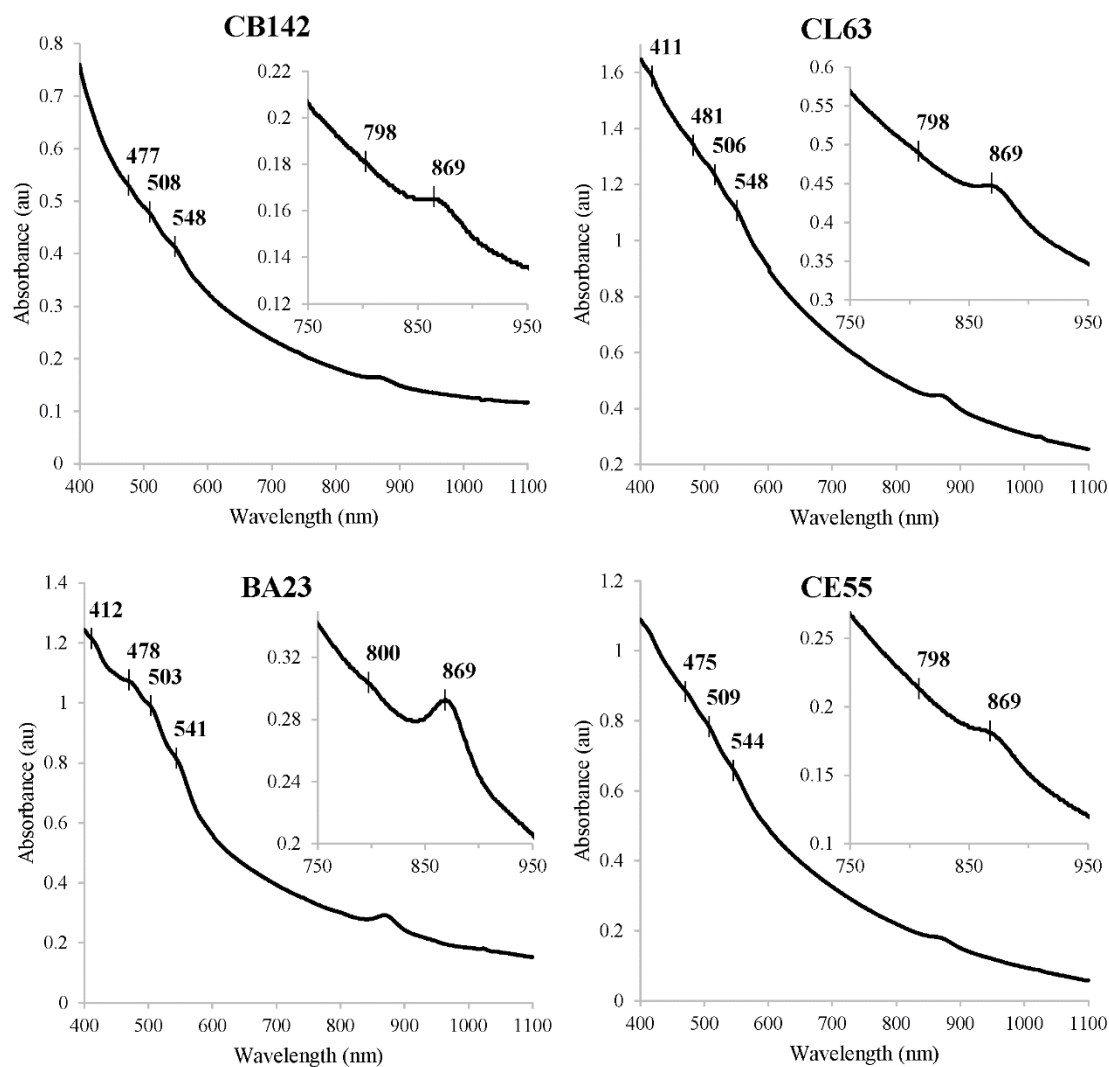


Fig. 4.1C. Whole cell spectra of each isolate. (A) *Erythrobacteraceae*, (B) *Sphingomonadaceae* and *Sphingosinicellaceae*, (C) *Methylobacteriaceae*, (D) *Rhodobacteraceae* and *Acetobacteraceae*, (E) Extra strains and type species absorbance trace from 380-1100 nm, with major peaks and shoulders of photosynthetic pigments marked.

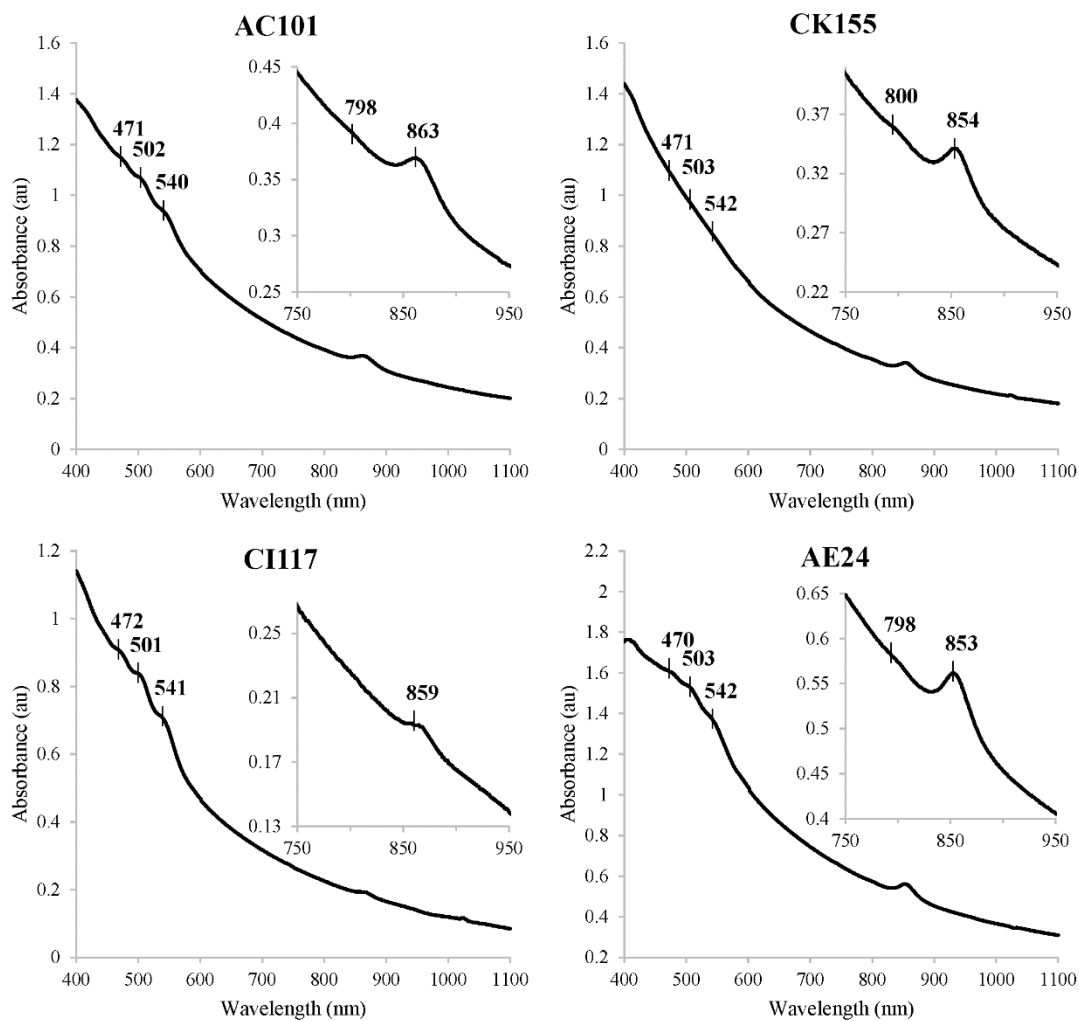


Fig. 4.1D. Whole cell spectra of each isolate. (A) *Erythrobacteraceae*, (B) *Sphingomonadaceae* and *Sphingosinicellaceae*, (C) *Methylobacteriaceae*, (D) *Rhodobacteraceae* and *Acetobacteraceae*, (E) Extra strains and type species absorbance trace from 380-1100 nm, with major peaks and shoulders of photosynthetic pigments marked.

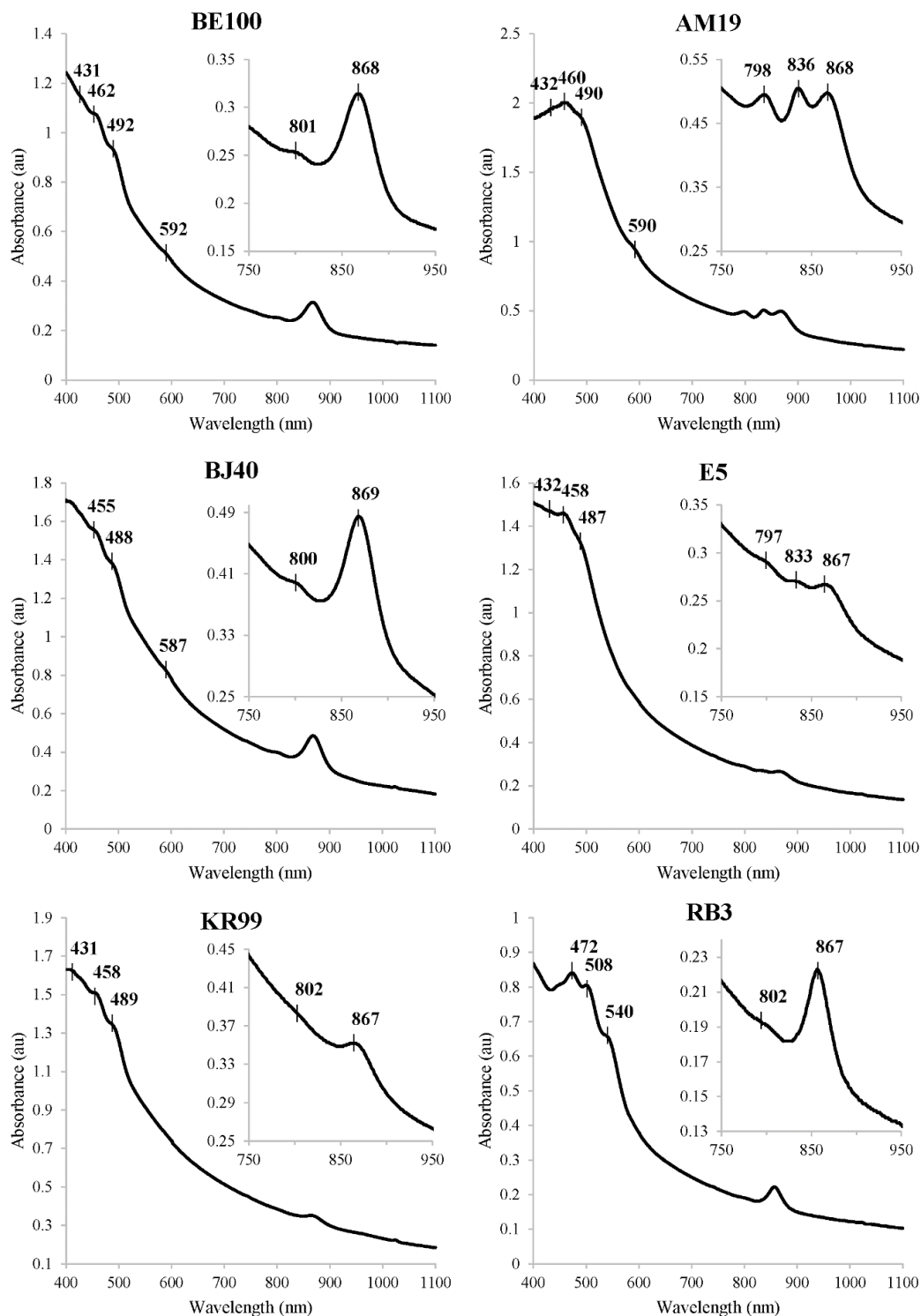


Fig. 4.1E. Whole cell spectra of each isolate. (A) *Erythrobacteraceae*, (B) *Sphingomonadaceae* and *Sphingosinellaceae*, (C) *Methylobacteriaceae*, (D) *Rhodobacteraceae* and *Acetobacteraceae*, (E) Extra strains and type species absorbance trace from 380-1100 nm, with major peaks and shoulders of photosynthetic pigments marked.

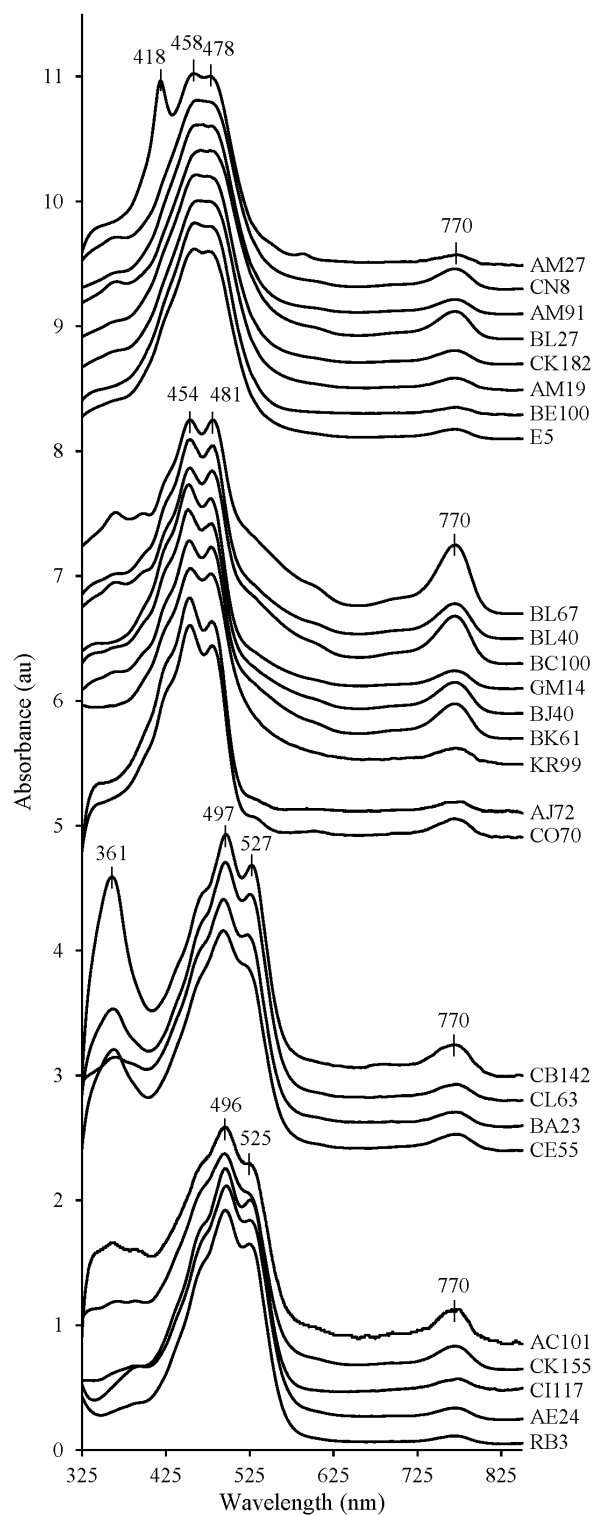


Fig. 4.2. Absorption spectra of AAP pigment extracts. Spectrum of 7:2 acetone:methanol extract from 7 days' growth cultures on RO. Each trace scaled at 475 nm to be at 1.5 au, and evenly displaced on graph for visualization.

variation, we defined them as ‘pinguis vibrioid’, due to their significantly enlarged size. In addition, AC101 was also atypical, having regular swelling and constrictions, making undivided cells look like beads on a string, or a pea pod (Fig. 4.3). Since it was a feature of single bacterium, and not caused by several unseparated cells in a filament, we chose to describe this as ‘moniliform’, previously used in microbiology terminology to reflect on “regular restrictions” (Aldred *et al.*, 1974).

All strains were gram-negative, while only some were motile via polar flagellum (Table 4.2). All strains were obligate aerobes, with none capable of anaerobic growth in the light or dark when cultivated with RO, or PNSM that had reduced acetate, malate, sulfide or thiosulfate as electron donors. None grew autotrophically in the presence of light, and fermentation of simple sugars such as glucose was not found. Aerobically, a range in enzyme activities, carbon source utilization, and antibiotic sensitivities was observed (Table 4.2). After reaching stationary phase of growth, most strains produced C_{16:0} or C_{16:1} as predominant fatty acid, while C_{14:0}, C_{17:0}, C_{18:0}, C_{18:1}, or C_{20:0} were among the top 5 detected (Table 4.3).

4.5.4. Growth ranges and pigment production

Stationary phase of growth was reached by all strains after a concurrent 7-day incubation at each temperature revealing a mesophilic tendency for most strains and a skewed distribution to tolerate cold, where most could grow at 16°C, but none tolerated higher than 45°C (Fig. 4.4). Many had optimum between 28-32°C, whereas CK155, CI117, and AE24 had most biomass at 32-37°C and only AC101 preferred 24°C. Carotenoids and BChl a were produced by all in coordination to their temperature preference, with more found in optimally grown cells, and less at either extreme (Fig. 4.4).

When pH ranges were assayed, “bell-shaped” optimum curves were not obtained. Rather, all strains could either grow at certain pH or they could not (Fig. 4.5). The lowest tolerated pH was 5.5, with three examples CO70, BC100 and AJ72 able to replicate at pH 11.5, the highest. These strains also displayed the widest range in pH tolerance, from pH 6 to 11.5, a 5.5 span. Carotenoids followed growth parameters, being produced proportionally to amount of protein (Fig. 4.5). In comparison, BChl a synthesis had 3 patterns. First, in the cells of CK182, AM27, CO70, BC100, AJ72, BK61, GM14 and BL67 it was produced at every pH the strain could grow. The second group included BL40, BL27, CN8, AM91, CB142, CL63, BA23, CE55 and AC101 with its synthesis only in acidic conditions (maximum at pH 5.5 – 6.5), while the final set of CK155, CI117 and AE24 made it under alkalinity of pH ~10.

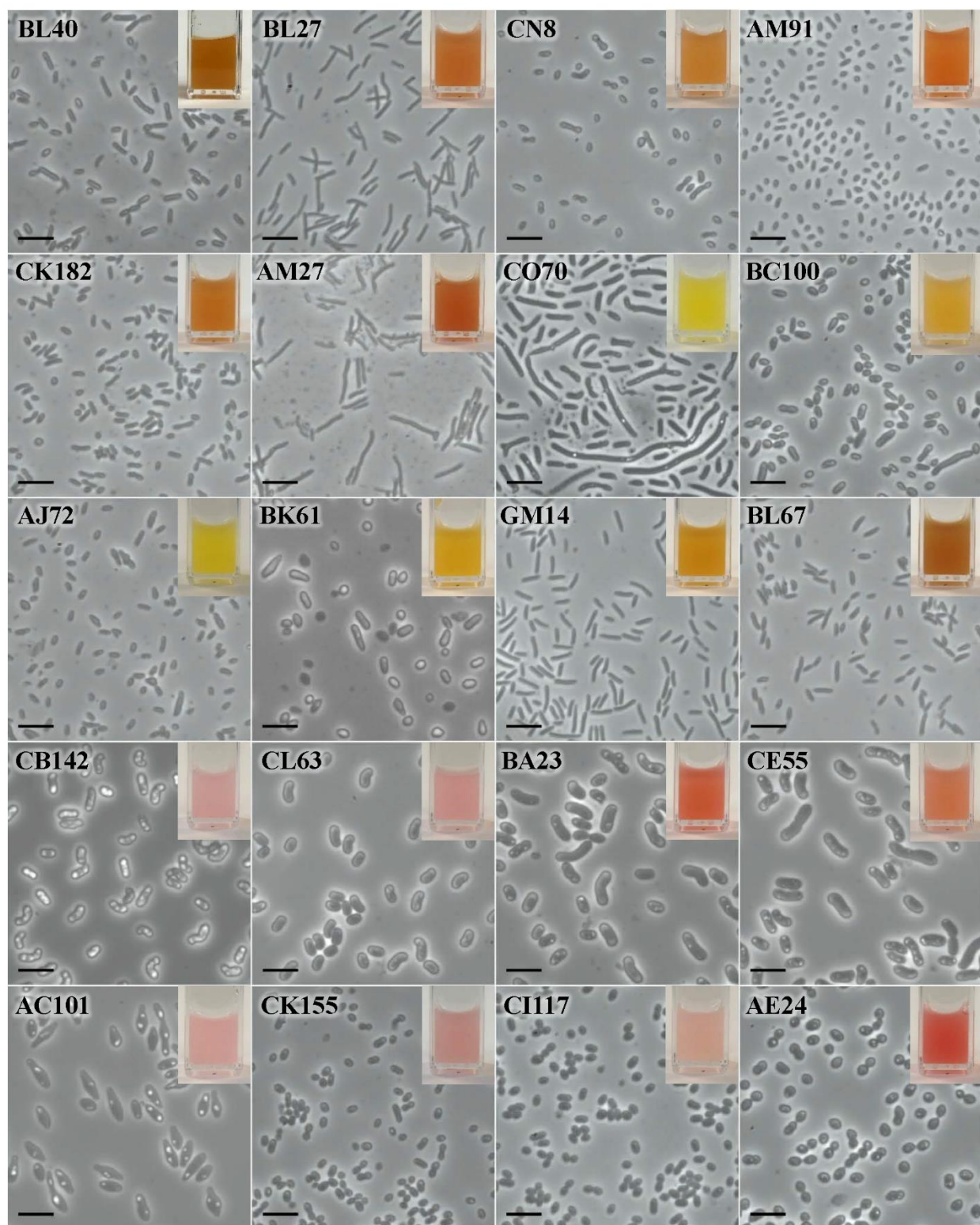


Fig. 4.3. Micrographs of 20 selected AAP species. Phase-contrast depiction of stationary-phase cultures after 7 days' growth on RO. Culture colouration in 10 mm cuvette normalized to 1.5 au absorbance at 660 nm as inset. Scale bar, 5 μ m.

Table 4.2. Physiological characteristics of AAP isolates, grouped by family. (1) *Erythrobacteraceae*, (2) *Sphingomonadaceae*, (3) *Sphingosinellaceae*, (4) *Methylobacteriaceae*, (5) *Rhodobacteraceae* and (6) *Acetobacteraceae* related strains.

	1								2					3		4		5		6			
Strains	BL40	BE100	BL27	CN8	AM91	CK182	AM19	AM27	AJ72	BK61	BL40	GM14	CO70	BC100	BL67	CB142	CL 63	BA 23	CE55	AC101	CK155	C117	AE24
Cell shape	Ro	Ro	Ro	Ov	Ov	Ov	RF	RF	Ov	Pl	Ro	Ro	Ro	Ov	Ro	PV	PV	PV	PV	Mo	Co	Co	CP
Colony Color	O	O	R/O	O	R/O	R/O	B/O	R/O	CY	Y/O	Y/O	Y/O	Y	Y	B	P	P	P	P	R/P	R/P	R/P	R/P
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	-	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	+	-
Enzyme Activities:																							
Catalase	+	+	W	+	+	+	-	+	+	+	W	+	W	+	-	+	+	+	+	W	W	W	W
Oxidase	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	W	+	+	W	+
Nitrate Reductase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of:																							
Agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	+	+	++	-	-	-	-	+	-	+	-	-	++	-	-	+	+	++	+	+	++	+	-
Starch	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Tween 60	+	+	++	+	+	+	+	+	++	+	++	+	++	+	+	-	-	-	-	-	-	-	-
Utilization of:																							
Acetate	+	+	-	+	-	+	+	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-
Bactopeptone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Butyrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Casamino Acids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Ethanol	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-
Formate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Fructose (D-)	-	+	-	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Glucose (D-)	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-	-	-	-	-
Glutamate (L-)	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-
Lactose (α-)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malate (L-)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
Methanol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-
Pyruvate	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
Succinate	-	-	+	-	+	-	+	-	-	-	-	+	+	-	-	+	+	+	+	-	-	-	-
Yeast Extract	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Antibiotic Sensitivity:																							
Ampicillin	S	S	R	S	R	S	R	R	S	S	S	S	S	S	S	R	R	R	R	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
Imipenim	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicillin G	S	S	R	S	R	R	R	R	S	S	S	S	S	S	S	R	R	R	R	S	S	S	S
Polymyxin B	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	R	R	R	S	S	R	R
Naladixic Acid	R	R	R	R	S	R	R	R	S	R	R	R	S	R	S	R	R	R	R	S	S	S	S
Streptomycin	R	R	R	R	R	R	S	S	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
Tetracyclin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Ro, Rod; Ov, Ovoid; RF, Rod Filament; Pl, Pleomorphic; PV, Punguis Vibroid; Mo, Moniliform; Co, Coccoid; CP, Coccoid Pair; O, Orange; R/O, Red/Orange; B/O, Brown/Orange; CY, Citron Yellow; Y/O, Yellow/ Orange; Y, Yellow; B, Brown; P, Pink; R/P, Red/Pink; +. Positive; -, Negative; W, Weak; S, Sensitive; R, Resistant.

Table 4.3. Fatty acid compositions of all isolates. 7 day-old RO cultures at stationary phase had content extracted and examined by GC similarity for pairwise comparisons. Grouped by (1) *Erythrobacteraceae*, (2) *Sphingomonadaceae*, (3) *Sphingosinellaceae*, (4) *Methylobacteriaceae*, (5) *Rhodobacteraceae* and (6) *Acetobacteraceae* related strains. Colour mapped based on abundance; high to low, Green → Yellow → Red. Type species indicated by light blue.

	1						2						3						4						5						6					
Strains	BL40	BE100	Cm8	Am91	Ckx82	Am19	Am27	E3	AJ72	BK61	RL440	GM14	P. nat	K99	COT0	BC100	BL67	R66-17	Cm42	CI 83	PA 23	CE55	AC101	CKX55	CM17	AE34	RE3									
Highest fatty acid composition (%)																																				
Primary	Cl6:1	Cl6:1	Cl6:0	Cl6:0	Cl6:0	Cl6:0	Cl6:0	Cl6:0	Cl6:1	Cl6:1	Cl6:0	Cl6:1	Cl6:1	Cl6:1	Cl6:0	Cl6:0	Cl6:1	Cl6:0	Cl6:0	Cl6:1	Cl6:0	Cl6:1	Cl6:0	Cl6:1	Cl6:0	Cl6:1	Cl6:0	Cl6:0								
	Cl6:0	Cl6:0	Cl4:0	Cl8:1	Cl7:0	Cl7:0	Cl8:1	Cl4:0	Cl8:1	Cl6:0	Cl8:0	Cl6:0	Cl6:0	Cl7:1	Cl8:0	Cl8:1	Cl6:0	Cl6:1	Cl6:1	Cl6:1	Cl6:0	Cl6:0	Cl6:0	Cl6:0	Cl6:1	Cl4:0	Cl6:0	Cl8:0								
	Cl8:1	Cl8:1	Cl8:1	Cl6:1	Cl4:0	Cl8:1	Cl6:0	Cl7:0	Cl6:1	Cl6:0	Cl7:0	Cl6:1	Cl7:0	Cl8:1	Cl6:1	Cl6:1	Cl7:0	Cl8:0	Cl8:1	Cl6:0	Cl8:1	Cl6:0	Cl8:0	Cl6:1	Cl6:1	Cl8:0	Cl8:1	Cl8:1								
	Cl8:3n3	Cl7:0	Cl6:1	Cl6:1	Cl7:0	Cl8:0	Cl6:1	Cl7:0	Cl6:1	Cl7:0	Cl8:1	Cl8:3n3	Cl7:0	Cl8:1	Cl6:0	Cl4:0	Cl4:0	Cl8:0	Cl4:0	Cl2:0	Cl2:0	Cl2:0	Cl6:1	Cl8:0	Cl6:1	Cl8:0	Cl8:1	Cl8:3n3								
	Entire Fatty acid composition (%)																																			
CB:0	ND	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.03	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND									
CL0:0	ND	0.2	0.0	0.1	0.0	0.1	0.1	0.1	ND	0.0	0.1	ND	0.2	0.2	0.1	ND	0.0	0.0	ND	0.2	0.1	0.2	0.2	ND	0.0	ND	ND	ND								
CL12:0	0.6	0.2	0.9	0.3	0.1	0.3	0.2	0.1	0.1	0.1	0.1	1.6	0.1	0.2	1.2	0.7	0.3	0.1	0.6	0.5	0.1	0.6	0.3	0.5	0.6	0.9	0.2	2.5								
CL14:0	1.0	1.2	6.1	14.2	2.5	2.0	1.2	10.9	16.2	4.9	0.9	1.6	1.6	2.1	0.5	9.7	7.8	2.5	3.4	0.5	0.5	1.2	2.2	1.2	1.2	1.96	0.5	1.4								
CL14:1	ND	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	ND	0.1	0.1	0.1	0.0	ND	0.0	0.1	ND	0.0	0.0	0.1	0.1	ND	0.1	ND	ND	ND								
CL15:0	0.2	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.2	1.4	0.4	0.3	0.3	0.2	0.1	0.3	0.4	0.2	0.2	0.4	0.1	0.2								
CL16:0	33.7	34.1	81.1	49.3	61.5	55.1	64.9	43.2	58.3	13.3	31.4	29.2	34.3	27.3	18.1	42.0	42.0	21.6	65.8	10.5	15.6	19.6	21.8	41.9	51.3	62.3	29.5	67.8								
CL16:1	0.5	ND	0.1	ND	ND	ND	ND	ND	ND	ND	0.6	ND	ND	ND	1.3	ND	0.2	0.8	1.0	ND	0.2	0.1	ND	0.2	0.1	ND	0.3	0.2	0.4							
CL61t	36.1	38.9	3.2	7.1	10.0	3.0	4.9	4.6	7.3	44.1	43.3	23.8	43.9	31.3	21.7	9.9	18.6	58.3	17.6	23.4	41.5	5.2	2.7	5.5	26.8	10.2	50.5	1.3								
CL17:0	1.4	7.6	0.8	6.8	4.8	12.6	14.2	10.1	7.1	6.8	7.4	2.7	7.5	11.6	10.5	2.2	5.1	5.6	0.3	1.2	0.7	1.6	1.1	0.3	0.3	0.3	0.7	0.7								
CL17:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	19.7	ND	ND	ND	ND	0.2	ND	ND	0.3	ND	0.3	ND	ND	ND								
CL18:0	18.8	5.7	4.9	3.7	4.3	8.7	3.9	5.3	1.8	0.7	5.1	27.5	2.6	10.0	4.3	21.1	3.3	3.9	7.5	25.9	16.9	22.1	16.8	6.9	3.3	2.8	15.3	18.9								
CL18:1	2.5	8.4	1.2	15.4	12.5	11.6	9.8	19.2	8.2	29.0	6.6	4.9	7.8	10.2	19.2	7.2	18.7	2.9	1.4	18.5	9.7	18.4	29.5	42.0	13.9	2.1	1.9	2.8								
CL18:2	0.5	0.3	0.1	0.2	0.1	0.5	0.2	0.3	0.1	0.1	0.1	0.7	0.1	0.4	0.5	0.6	0.1	0.1	0.2	0.9	1.0	1.2	0.9	0.0	1.1	0.1	0.2	0.4								
CL18:3n6	ND	0.1	0.1	0.1	0.0	ND	0.0	0.1	0.0	0.2	0.0	ND	0.0	0.1	ND	ND	0.1	0.0	0.4	ND	0.2	ND	ND	0.5	ND	ND	ND	ND								
CL18:3n3	4.2	0.0	0.6	0.4	0.0	0.0	0.0	0.0	ND	0.0	0.0	6.7	0.0	0.1	ND	3.6	0.0	0.0	2.5	0.0	0.0	1.0	0.1	0.1	0.1	0.1	0.5	2.8								
CL20:0	0.3	0.1	0.0	ND	0.0	0.1	0.0	0.1	ND	0.0	0.1	0.4	0.0	0.1	0.2	0.3	0.0	0.1	0.1	13.9	10.5	13.7	9.5	1.1	0.0	0.1	0.2	0.3								
CL20:1	0.1	0.1	0.0	ND	0.0	3.0	1.7	0.0	0.0	ND	2.8	0.0	0.0	3.5	ND	1.8	1.8	ND	1.4	0.9	5.3	2.8	ND	ND	ND	ND	ND	ND								
CL20:2	ND	ND	ND	0.3	ND	0.2	0.0	0.1	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND	0.1	ND	0.1	1.5	ND	ND	ND	ND	ND	ND								
CL20:3n6	ND	ND	ND	ND	ND	ND	0.1	0.0	ND	ND	ND	0.2	0.1	ND	ND	0.1	ND	0.1	ND	ND	0.3	0.5	ND	ND	ND	ND	ND	ND								
CL20:4	ND	0.1	0.2	0.1	0.5	ND	0.1	0.2	0.1	0.1	0.4	0.2	0.1	0.5	0.1	0.1	0.4	0.1	0.0	0.0	0.7	2.1	3.1	0.0	0.1	0.3	0.3	0.2								
CL20:3n3	ND	0.1	ND	0.1	0.1	0.0	0.0	0.0	ND	0.0	1.2	ND	0.0	0.1	ND	ND	ND	ND	ND	ND	ND	0.2	0.0	0.0	0.0	0.0	ND	ND								
CL20:5	ND	1.4	ND	0.7	1.2	2.1	0.1	2.7	0.3	0.2	0.6	0.1	0.5	4.3	1.7	ND	1.4	1.8	0.0	0.8	0.4	6.4	3.6	0.0	0.1	0.8	ND	0.8								
CL22:0	ND	0.0	0.0	ND	ND	ND	ND	ND	ND	0.0	ND	ND	ND	ND	ND	ND	1.4	1.8	0.0	0.2	0.1	0.1	ND	ND	0.1	ND	ND	ND								
CL22:1	ND	1.3	0.6	1.0	1.8	0.1	2.5	0.2	0.2	1.5	ND	0.6	3.6	1.4	ND	ND	0.1	0.0	ND	0.2	0.1	0.1	ND	ND	0.1	ND	ND	ND								
CL22:2	ND	0.0	0.0	ND	ND	0.0	0.0	ND	0.0	0.1	ND	0.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.5	0.7	ND	ND	ND	ND	ND								
CL22:4	ND	ND	ND	0.0	0.1	ND	0.0	ND	0.0	0.0	ND	0.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.4	ND	ND	ND	ND	ND	ND								
CL22:5n3	ND	0.0	ND	ND	ND	ND	ND	ND	0.1	0.0	ND	0.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0	0.3	ND	ND	ND	ND	ND								
CL22:5n3	ND	0.0	ND	ND	ND	ND	ND	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0	0.0	ND	ND	ND	ND	ND								
CL24:0	ND	0.0	ND	ND	ND	ND	ND	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0	0.2	0.2	ND	0.1	ND	ND	ND								
CL24:1	ND	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	ND	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	ND	0.0	0.0	0.1	ND	0.1	ND	ND								

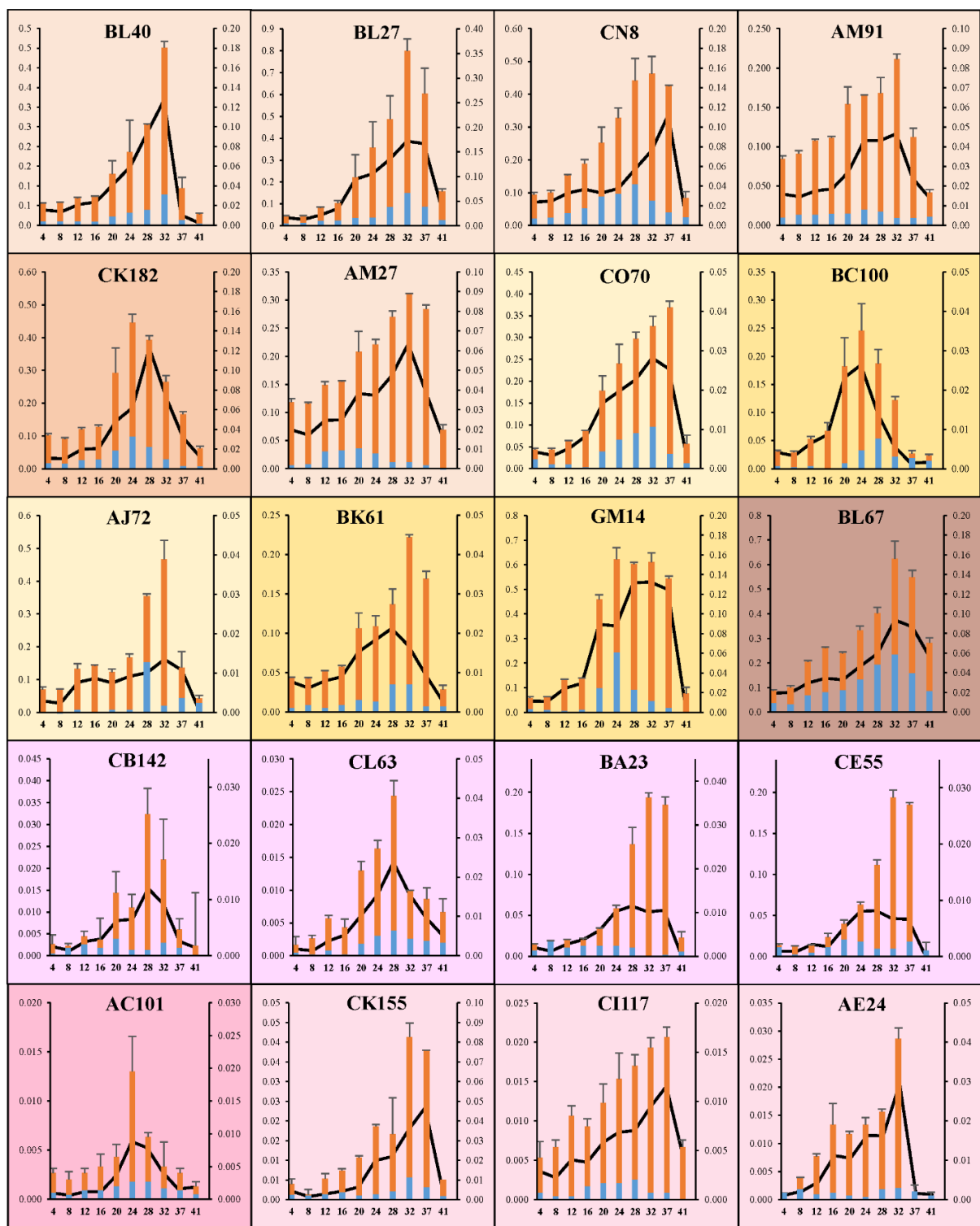


Fig. 4.4. AAP growth and pigmentation at different temperatures. Relative levels of produced pigments at various temperature (X-axis) included extracted carotenoid concentration, orange (left Y-axis measured at 450 nm); BChl a level, blue (right Y-axis measured at 770 nm); total cellular protein trace depicted behind in black; each after 7 days of growth.

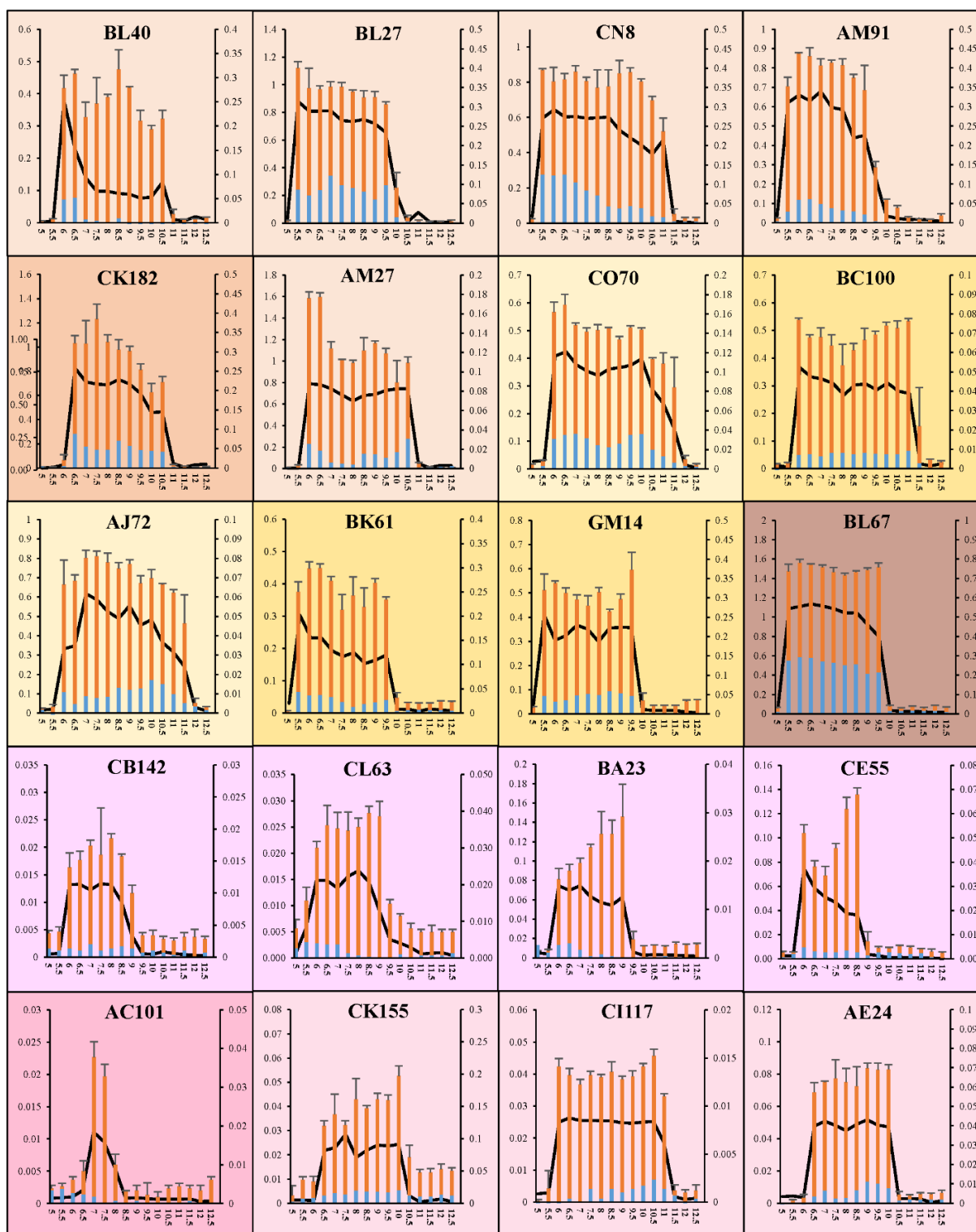


Fig. 4.5. Influence of pH on AAP growth and pigmentation at defined increments. Relative levels of produced pigments at various pH (X-axis) included extracted carotenoid concentration, orange (left Y-axis measured at 450 nm); BChl a level, blue (right Y-axis measured at 770 nm); total cellular protein trace depicted behind in black; each after 7 days of growth.

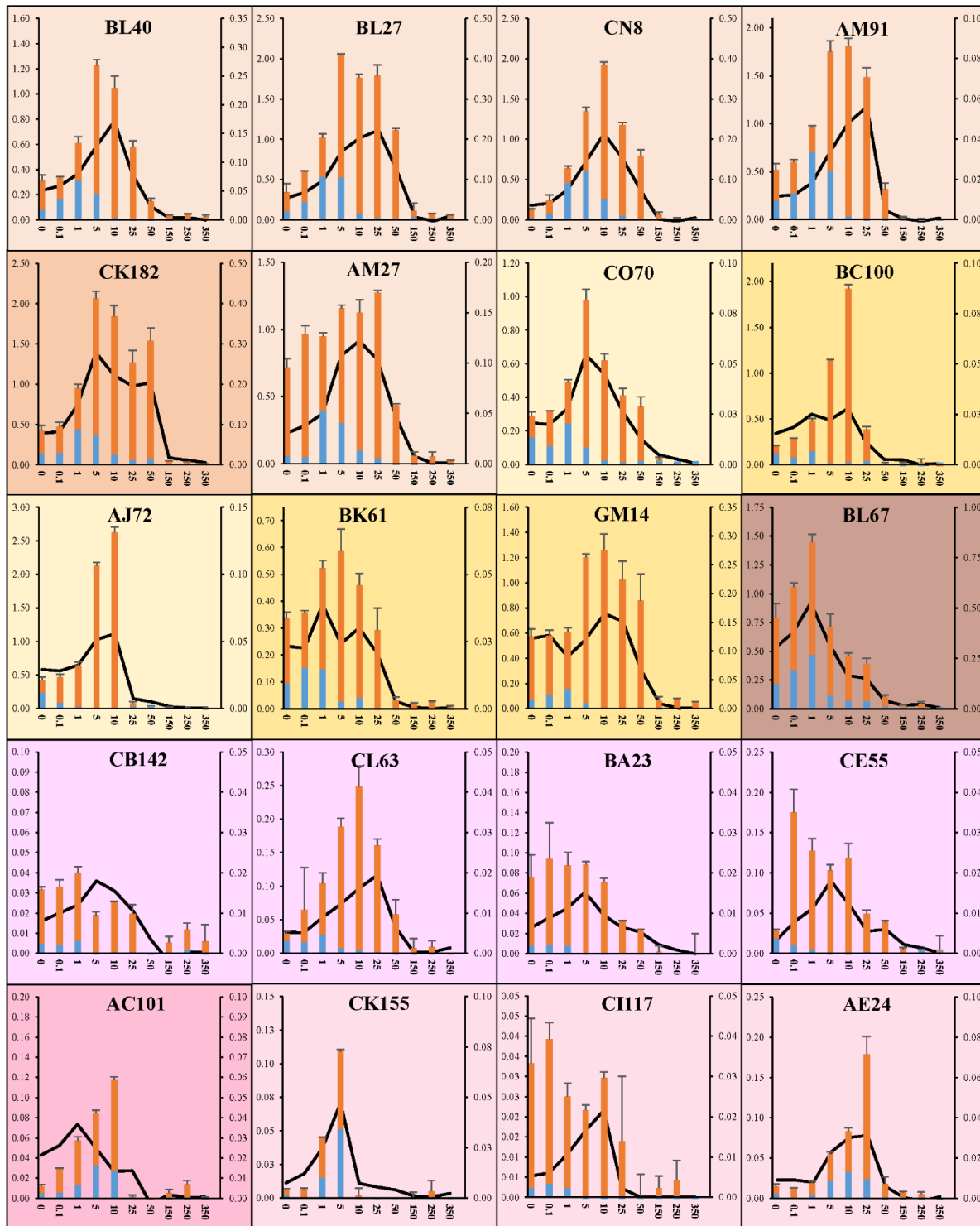


Fig. 4.6. Oligotrophic and copiotrophic limits of AAP pigmentation and growth. Relative levels of produced pigments at various nutrient concentrations (X-axis, g/L) included extracted carotenoid concentration, orange (left Y-axis measured at 450 nm); BChl a level, blue (right Y-axis measured at 770 nm); total cellular protein trace depicted behind in black; each after 7 days of growth.

The effect of complex organic carbon availability tested via increasing the amount of yeast extract showed that most strains had optimal growth at higher than 1 g/L, the amount typically provided in standard RO (Fig. 4.6). Inhibition was recorded only at levels near saturation (Fig. 4.6). While a few strains including BL27, AM91, and CL63 preferred up to 25 g/L, most grew best at 5-10 g/L, with only BL67 and AC101 requiring 1 g/L. No isolates grew better when complex carbon was less than in typical RO composition. Carotenoids corresponded to protein production for all strains (Fig. 4.6). While a few strains also had BChl a tracing growth, including BL67, AC101, CK155, CI117, and AE24, the rest preferentially produced their primary photosynthesis pigment under relatively oligotrophic conditions, when compared to carotenoids. By the majority of strains, BChl a was synthesized at its highest levels when supplemented with relatively reduced organic content, 0.1-1 g/L of yeast extract.

Considering metal(loid) tolerances, an impressive tolerance to over 1000 µg/mL tellurite was observed for strains AM91, CK182, AM27, BK61, BJ40 and GM14 (Table 4.4). Selenite tolerance above 1000 µg/mL was also detected in several isolates including BE100, BL27, AM19, AM27, BK61, GM14, and AE24. Furthermore, most strains that had MIC over 500 µg/mL had evidence of metal(loid) oxide reduction, by either the blackening or reddening of tellurite and selenite supplemented cultures, respectively.

4.5.5. Genetic identification of AAP strains via 16S rRNA gene sequencing

Partial 16S rRNA gene sequences of each isolate were compared to database records relating to 20 type species hailing from an assortment of α -proteobacterial families (Table 4.1). These included 5 from *Erythromonadaceae*; 6, *Sphingomonadaceae*; 1, *Sphingosinicellaceae*; 4, *Methylobacteriaceae*; 1, *Rhodobacteraceae*; and the final 3, *Acetobacteraceae*. While some had high levels of 16S rRNA gene similarity to described species (up to 99.86% for BJ40 to *B. natatoria*), others were lower, such as AC101 with 96.16% to *Rubellimicrobium rubrum*, potentially branching from any known representatives (Fig. 4.7). In addition, while many strains related to known AAP species, several were strains of bacteria previously identified as heterotrophs, including AJ72, AC101, CO70, BC100, CB142, BA23, CE55, CK155, CI117, and AE24, as highlighted in Fig. 4.8.

Table 4.4. Metal(loid) tolerance levels to tellurite and selenite of lake AAP isolates. Blackening or reddening of tubes indicative of reduction to tellurium or selenium, respectively, denoted.

Strains	BL40	BE100	BL27	CN8	AM91	CK182	AM19	AM27	A172	BK61	BL40	GM14	CO70	BC100	BL67	CB142	CL63	BA23	CE55	AC101	CK155	CL117	AE24
MIC (Minimum Inhibitory Concentration)																							
Tellurite (µg/mL)	250	250	1000	500	>1500	2000	500	1500	500	>1500	1500	>1500	1000	250	500	250	1000	50	50	250	75	100	25
oxide reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	w	w	+	-	-	-
Selenite (µg/mL)	100	1500	>1500	500	250	1000	1500	1500	250	>1500	500	>1500	250	100	100	250	500	500	1000	250	1000	750	>1500
oxide reduction	+	+	+	+	w	+	+	+	w	w	w	w	w	w	-	w	w	w	+	+	w	+	+

Positive, +; Negative, -; Weakly positive, w.

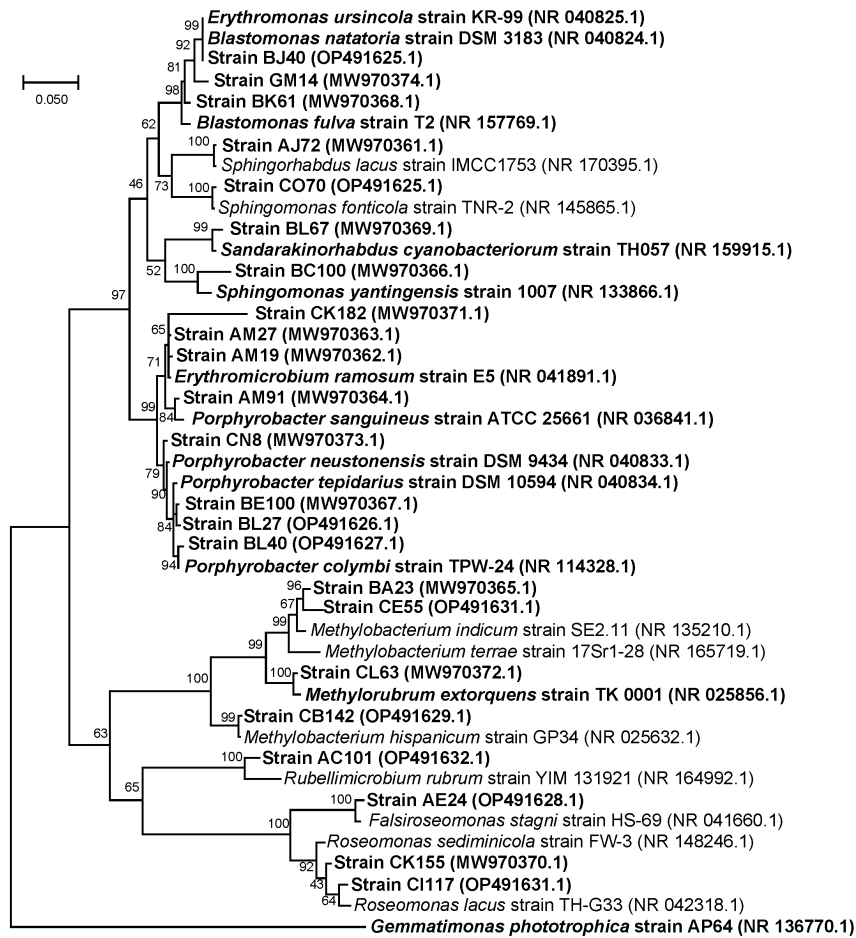


Fig. 4.7. AAP 16S rRNA gene similarity phylogenetic tree to nearest type species. Maximum Likelihood method and Tamura-Nei model used. Branch length measured number of substitutions per site. AAP (**bold**); heterotrophs (slender script).

4.6. Discussion

4.6.1. AAP isolation and identity

To characterize strains, it was reasonable to group them by genetic relatedness, once the presence of particular LH and RC complexes were detected in aerobically grown cultures (Table 4.1). The collection was purified from both limnetic and littoral locations of Lake Winnipeg and several medium compositions including oligotrophic and classical rich organic medium, OM and RO, respectively, in addition to a copiotrophic potato-broth agar, or R2A which had organic content between OM and RO (Chapter 2). Furthermore, tellurite containing RO medium (RO_T) also yielded a few examples, including strains CK182, AM27, AJ72 and BC100. Taken together, this suggested that AAP had a range of specific organic carbon utilization profiles, and that 100 µg/L tellurite provided a modicum of selective pressure as some strains survived the toxic metalloid oxide, while other bacterial growth was inhibited. In general, *Erythrobacteraceae*, *Sphingomonadaceae*, and *Sphingosinellaceae* relatives were isolated from shorelines, whereas *Methylobacteriaceae*, *Acetobacteraceae*, and *Rhodobacteraceae* were purified mostly offshore, potentially indicating on their habitat preference (Table 4.1).

4.6.2. Pigment analysis

The initial identification of aerobically grown strains as AAP was done by analyzing *in vivo* aerobic production of LH and RC complexes, which were best detected after a weeklong incubation in the dark, as all strains had matured into stationary phase (Fig. 4.1, Table 4.1). While each strain had a peak at ~ 799 nm with a minor 1-2 nm deviation and could be tentatively attributed to RC, absorption between 810-900 nm matched LHI and LHII features. Furthermore, strains that genetically related to a particular family all shared similar trends in absorbance maxima, suggesting common RC and LH configurations among the taxon (Table 4.1). Particularly, *Erythrobacteraceae* relatives had LHI with peaks between 867-869 nm, common in the family (Yurkov and Beatty, 1998). In addition, AM19 and AM27 produced the characteristic 800-(834-835) nm LHII indicative of their closest freshwater AAP relative *E. ramosum*. Unusually, the distantly related BL27 also had an 835 nm peak of presumably LHII (Fig. 4.7, 4.8; Table 4.1), suggesting this complex may have been present in other members of the family and was later evolutionarily lost, or recently acquired via horizontal gene transfer (HGT). Strains BK61, BJ40 and GM14 related to known AAP genus *Blastomonas* of *Sphingomonadaceae*, lacked LHII, and had the anticipated LHI at 867-870 nm. The singular example of the recently described *Sphingosinellaceae*, BL67, also had a typical 869 nm LHI, but was notable as it also contained an 855 nm peak indicative of LHII. While similar LHII have been identified in several anaerobic

anoxygenic purple bacteria (Csotonyi *et al.*, 2011), this is the first observation of its production aerobically by any known AAP. Further interesting spectra were recorded from the only *Rhodobacteraceae* isolate AC101, and the far branching *Sphingomonadaceae* strains AJ72 and CO70 that had blue-shifted 862-863 nm LHI. Similar absorption range had only been found among acidophilic AAP belonging to *Acetobacteraceae* species *Acidiphilium rubrum* (Yurkov and Beatty, 1998), and recently documented new members of *Hyphomonadaceae*, *Photocaulis* (Kuzyk *et al.*, 2022a). While *Methylobacteriaceae* strains CB142, CL63, BA23, and CE55 all had typical 869 nm LHI, the most redshifted complex was in BC100 with a peak at 875 nm. The *Acetobacteraceae* isolates CK155, CI117, and AE24 each had 852-854 nm, the strongest blue-shifted LHI. Every variation was specific to the genetic group, implying evolutionary descent with modification.

The comparison of spectra from extractable pigments further correlated AAP isolates to their genetic families (Fig. 4.2). First, all contained BChl *a* as their primary pigment for photosynthesis, indicated by a maximum at 770 nm in 7:2 acetone:methanol. Since no other BChl was found, the range of maximal peaks from 852-875 nm detected in whole cells was likely a result of conformational shift in the protein composition of each RC and LH complex. However, additional analysis of the blue shifted *Acetobacteraceae* complexes, as well as from *Sphingomonadaceae* strains AJ72 and CO70 or *Rhodobacteraceae* isolate AC101 may show whether the shift is due to an alternative central cation in the molecule of BChl *a*, such as Zn found in *Acidiphilium rubrum* (Wakao *et al.*, 1996).

Second, carotenoids absorbing between 400-600 nm revealed a few important trends. All *Erythrobacteraceae* cell extracts were orange; *Sphingomonadaceae*, yellow-brown; *Sphingosinicellaceae*, brown; *Methylobacteraceae*, bright pink; *Acetobacteraceae*, pastel pink; and *Rhodobacteraceae*, fuchsia pink. Carotenoid absorbance maxima were similar for all members of a family and matched their grouped colourations, indicating family-specific assortment of secondary pigments. The slight variability in whole-cell hue of a few related strains of *Methylobacteraceae* BA23 and CL63, was likely due to differences in relative expression of specific carotenoids from the set (Fig. 4.3), as no novel peaks were observed for either strain. Chromophores such as cytochromes absorbing between 300-400 nm also revealed similarities between members, with *Methylobacteriaceae* having a particular peak at 361 nm worth investigating. A few exceptions were found between the likeness of each group, where AM27 had a peak at 418 nm, not found in any other *Erythrobacteraceae* from Lake Winnipeg, but published for related marine AAP *Erythrobacter litoralis* (Yurkov *et al.*, 1994) potentially representing a novel molecule reminiscent to known cytochromes.

Some families had pigment profiles resembling that of their closest genetic neighbors. *Sphingosinellaceae* strain BL67 was akin to *Sphingomonadaceae* for its peaks between 425-525 nm. However, it also absorbed between 325-425 and 525-625 nm, which only corresponded to a few known members of *Sphingomonadaceae*. These additional peaks could likely explain both its striking deep-brown appearance, and the potential for related *Sphingomonadaceae* AAP to become brown under some specific conditions. While CO70 and AJ72 had carotenoid peaks at 454 and 481 nm similar to other *Sphingomonadaceae* and *Sphingosinellaceae*, they specifically lacked any absorbance in the 525-625 nm range, which correlated to their definitive citron-yellow colouration, suggesting both may not have the ability to produce browning-pigments like the other strains. Furthermore, the *Rhodobacteraceae* representative AC101 had a pigment profile that resembled the genetically similar *Acetobacteraceae* group (Fig. 4.2).

4.6.3. Microscopy and physiology

Similar to pigments, morphological patterns were apparent after evaluating genetic relatedness (Fig. 4.2,4.3). *Erythrobacteraceae*, *Sphingomonadaceae* and *Sphingosinellaceae* were all ovoid to rod shaped, with a few important deviations. Strains BL27, AM27, and AM19 each developed slender elongated rods, the same group that had peaks at 835 nm, of presumably LHII. CO70 had rare branching morphology, comparable to cells of related AAP *Sandaracinobacter sibiricus* (Yurkov and Beatty, 1998). Every new strain related to *Methylobacteriaceae* was enlarged and vibrioid, defined as pinguis vibrioid. *Acetobacteraceae* cells also grouped together as coccoid shaped, resembling some described type species (Yurkov and Gorlenko, 1992). AE24 atypically formed coccoid pairs, a feature not published for any AAP previously. Finally, AC101 relative of *Rhodobacteraceae* had the most interesting cells with regular restrictions, which caused them to have a moniliform appearance. Such arrangement was also new for AAP, and deserves future morphological analysis. In addition to overall exciting morphology, several strains had intracellular cytoplasmic accumulations (CO70, CB142, BA23, CE55, AC101 and AE24), indicative of poly-hydroxy-alkanoates, reported for some AAP species (Yurkov and Beatty, 1998).

In addition to nutrient storage, several growth characteristics of strains were common within families (Table 4.2). While half of *Erythrobacteraceae* was motile, the filamentous-like examples related to *E. ramosum* were not. All *Sphingomonadaceae* and *Sphingosinellaceae* were motile, except CO70, which again formed “filamentous” branching morphology. This might suggest that species which evolved elongated forms purposely forewent any motility, and their lengthy shape may have an important role in the habitat.

All *Methylobacteriaceae* were motile with polar flagella, consistent as their feature among the family members. In comparison, every *Acetobacteraceae* strain was incapable of locomotion, again having uniform coccoid appearances. Finally, the moniliform *Rhodobacteraceae* AC101 had enlarged cells, which in tandem with the previous assessment of filamentous shape, fit well with its lack of motility. Other important trends differentiating families included the special capability of *Acetobacteraceae* isolates to reduce nitrate, whereas only *Erythrobacteraceae*, *Sphingomonadaceae* and *Sphingosinellaceae* hydrolyzed Tweens. Several carbon sources were utilized only particular families, such as butyrate by *Erythrobacteraceae*, *Sphingomonadaceae* and *Sphingosinellaceae*, whereas methanol was specifically consumed by *Methylobacteriaceae*. *Acetobacteraceae* and *Rhodobacteraceae* strains did not replicate when supplied with simple carbon compounds, with the former meagerly sustainable by yeast extract, and the later incapable of growth when supplied with any sole nutrient. These findings suggested that the *Erythrobacteraceae*, *Sphingomonadaceae*, *Sphingosinellaceae*, and *Methylobacteriaceae* were individually more metabolically diverse than *Acetobacteraceae* and *Rhodobacteraceae* which require specific, yet currently unknown substrates to thrive.

Antibiotic resistances also provided family dependent patterns, where most isolates had a sensitivity to a majority of the drugs tested. As an additional feature, cellular fatty acid profiles revealed all AAP to have C_{16:0} and C_{16:1} as predominant lipids, 61.5±16.4% of their averaged composition (Table 4.3). In particular, *Erythrobacteraceae* and *Acetobacteraceae* had C_{16:0} at 50-60%, when all others had below 40%. *Sphingomonadaceae* and *Sphingosinellaceae* instead produced more C_{16:1}, with the later up to 58.3%, while all other bacteria had C_{16:1} below 30%. *Methylobacteriaceae* were enriched for C_{18:0} and C_{18:1}, totaling ~20%, where *Rhodobacteraceae* AC101 had 42%, which was also diminished in each other family. In addition, *Methylobacteriaceae* contained C_{20:0} at ~10%, altogether absent in other AAP.

4.6.4. Growth ranges and pigment production

While detailed knowledge of pigment synthesis and nutrient utilization were important features to determine individually, we were also curious in monitoring both together, to better understand how AAP promoted photosynthesis during growth at a range of different conditions. While some particular factors enhanced total yield of bacteria, primary and secondary pigment synthesis could be stimulated at either optimal or under stress conditions. The change in temperature, pH, and complex carbons delivered both limitations and stimulations for growth, carotenoid and BChl a content depending on strain. Specifically, while most AAP had highest density when incubated at 28-32°C, they were also found to have a growth

preference that takes on a skewed-bell-curve shape over a range that provided capability of minimal replication at 4°C (Fig. 4.4). The potential to persist and moderately thrive at colder conditions made sense, as conditions in Lake Winnipeg fluctuated from ~1 to 25°C every year (Chapter 1), and bacteria from such a habitat would evolve such a tolerance (Price and Sowers, 2004). This also then corresponded to the high presence of AAP in the spring season, as they tolerated and thrived in the slow-to-warm waters prior to the peak of heat during summer (Chapter 2). Curiously, in laboratory they preferred a warmer temperature for optimal growth than found in the lake, but similar phenomenon of mismatching has been documented for many environmental isolates (Nedwell, 1999). Furthermore, each was thermo-intolerant with sharp inhibition near 45°C, as such level of heat was altogether absent in the lakes hydrological history (MARD, 2020). Regarding pigments (Fig. 4.4), all strains produced significantly more carotenoids than BChl *a*, typical for AAP (Yurkov and Beatty, 1998). Moreover, both carotenoids and BChl *a* content directly traced optimal growth and were produced in correlation to the amount of biomass. Temperature therefore had a generalized impact on AAP life as a whole metabolic system inhibitor or stimulator, that did not independently promote pigment production under stress conditions.

Different strategies were noted after challenging isolates to distinct pH (Fig. 4.5). In general terms, the growth of AAP at various proton concentrations acted as an “on-off” switch, where AAP either survived certain pH or they did not. Exemplified by *Erythrobacteraceae*, *Sphingomonadaceae*, *Sphingosinicellaceae* and *Acetobacteraceae* relatives, pH ~6-11 allowed each strain to reach the relative maximal density uninhibited, whereas above and below this range, each completely inhibited replication. *Methylobacteriaceae* and *Rhodobacteraceae* were different, and had more “bell curve” appearing growth patterns. This distinction suggested family dependent mechanisms for pH tolerance. The first allowed for growth unaffected at different pH with high levels of tolerance, until they reached a point of no persistence possible. The second had a weaker regulatory system and was only capable of combating pH gradients to a degree, being less effective at the boundaries of tolerance. Of note, since optimal growth curves did not have obvious peaks in most cases and instead reached a plateau, the medium tolerated pH was determined as between 7.5 and 8.5. Such marginally alkaline values were identical to the pH of their ecosystem Lake Winnipeg, which reflected on some habitat adaptation (Chapter 2). Carotenoid production consistently followed growth, only being affected by optimal conditions similar to the influence of temperature. BChl *a* regulation was a different story, with 3 characteristic patterns recognized. The *Erythrobacteraceae* strains CK182 and AM27 related to *E. ramosum*, as well as every *Sphingomonadaceae* and *Sphingosinicellaceae* example (AJ72, BK61, BJ40,

GM14, CO70, BC100 and BL67) had no particular impact from pH, having BChl *a* produced whenever the cells were the healthiest, similar to carotenoids. The pattern was different for the distantly related *Eythrobacteraceae*, *Methylobacteriaceae* and *Rhodobacteraceae* strains (BL40, BL27, CN8, AM91, CB 142, CL63, BA23, CE55 and AC101), which had BChl *a* promoted predominantly under acidic conditions. In comparison, the *Acetobacteraceae* CK155, CI117 and AE24 were the only group that made primary pigments mostly at alkaline pH of 9.0-10.5. Taken together, certain groups of AAP modulated their photosynthetic abilities, or at least BChl *a* production, to favour particular factors. As Lake Winnipeg has been typically around pH 8.3, it is not clear why some AAP preferred higher or lower values. Perhaps certain strains have microenvironments in the water column, attached to particles (Koblížek, 2015), or other microbes that may create and support required conditions.

The third major set of growth experiments tested the regulation by available complex organic nutrients (Fig. 4.6). The copiotrophy vs. oligotrophy of AAP has come into question as they lack autotrophy and require organics to thrive (Yurkov and Beatty, 1998), but the ideal amount of nutrients had yet to be established. Among other findings, several recent studies can be grouped by their use of diluted R2A for the isolation of AAP (Zervas *et al.*, 2019; Kopejtká *et al.*, 2021). However, in the contemporary analysis of Lake Winnipeg's microbial communities, although more heterotrophs were culturable using oligotrophic OM, higher numbers of AAP could be collected on a richer RO medium (Chapter 2). Here, complex organics stimulated them over a "bell-curved" stylized pattern of growth, where both minimal and maximal concentrations suppressed replication. While a lack of organics likely starved the AAP, we suggest they were only inhibited by the increased osmolarity at higher concentrations, and not necessarily by the toxic effect of excess nutrients. However, most grew best at 5-10× more nutrients than present in the RO composition, with only the *Sphingosinicellaceae* and *Rhodobacteraceae* strains BL67 and AC101, respectively, preferring 1× (regular) amounts for best growth. Similar to the effect of both pH and temperature, carotenoid synthesis in all strains followed maximal growth, which peaked when cellular replication did, averaging at higher than typical RO levels of 1 g/L yeast extract. This aligned well with known information that potato-broth agar and other high nutrient containing media cause several AAP to develop vibrant colours (Yurkov and Beatty 1998). What was novel here however, was that BChl *a* synthesis did not correlate with optimal growth, but instead had highest yield at nutrient-deprived conditions for all strains, with organics from 0.1 to 1 g/L, at or below RO concentrations. Here it is thus reported that while many AAP may prefer to grow in nutrient rich environments, they activate expression of their photosynthetic components when organics are limited. These

findings also move forward our understanding as to why several AAP were initially isolated on relatively diluted R2A (Zervas *et al.*, 2019; Kopejtká *et al.*, 2021), as it would promote isolates to produce detectable RC and LH complexes. While RO and other complex media were enhancing growth, it was only after laborious testing and screening with nutrient limitations, each was confirmed as physiologically belonging to AAP. Taken together, AAP metabolism appears directly linked to organic availability. Under starvation conditions they up-regulate synthesis of photosynthetic membranes to produce additional ATP, thereby reducing the breakdown of organic compounds for respiration, and only using them as building blocks for anabolism. When bulk nutrients are available, photosynthesis can be suppressed, and cells instead perform nutrient-based chemosynthesis similar to strict heterotrophs.

Future analysis of specific carbon compounds could show how concentration effects utilization and photosynthesis stimulation. As the *Rhodobacteraceae* and *Acetobacteraceae* strains were not assimilating typical sugars (Table 4.2), the discovery of their organic preference may yield similar results as seen in strains from other families, where low levels stimulate BChl a production, and high concentrations promote growth, but repress BChl a.

As a final goal, growth over a range of tellurite and selenite concentrations was monitored, as AAP have been found strikingly tolerant (Yurkov and Beatty, 1998; Maltman and Yurkov 2015), and pigments promoted when challenged against metalloid oxides (Csotonyi *et al.*, 2014). While Lake Winnipeg had negligible metal content in water (<1ppb Te and Se), 100 µg/mL TeO_3^{2-} added to media was partially selective for AAP (Chapter 2, 3). Therefore, isolates from such a mundane environment were examined to see if they expressed high levels of tolerance or sensitivity.

Overall, oxide tolerance matched phylogenetic profiles, as the most tolerant Lake Winnipeg AAP related to known highly tolerant bacteria, forming similar groups (Table 4.4). *Erythrobacteraceae* associated to *E. ramosum* were the most tolerant to both metalloid oxides, followed by relatives of *B. ursincola*. As both type species were extremely tolerant (Yurkov *et al.*, 1996), this strongly supported that all bacteria in clades perform similar metal transformations. Furthermore, the blackening/reddening of tubes confirmed metalloid oxides reduction, likely with similar strategies as in the type species (Maltman and Yurkov, 2015). In comparison, no methylotrophic, *Rubellimicrobium* or *Roseomonas* related AAP had been previously tested for tellurite or selenite tolerance, therefore no type strains were comparable. Here, most were found to have minimal tolerance to tellurite, having MIC ~100 µg/mL. While higher than 1 µg/mL, which was previously

considered toxic to most bacteria, these groups lacked the tolerance profiles of *Erythrobacteraceae* and *Sphingomonadaceae*, and also had minimal or absent culture colour changes, indicating on limited reduction to elemental Te. However, high levels of tolerance to selenite with reduction to elemental Se detected was found among the *Roseomonas* strains, suggesting all shared a similar mechanism for this particular transformation. The conversion of one metalloid oxide and not the other supports the notion that some enzyme specificity is present for this resistance mechanism. Still regarding Se, the *Sphingomonas*, *Sphingorhabdus*, and *Sphingosinicellaceae* relatives all inversely had minimal influence and low tolerance.

Results above strongly suggest that AAP perform metal transformations in their natural environment. As some enzyme specificity was detected, future analysis of multiple metals will determine AAP preference and could associate this wide spectrum of function to their niche. Since several metals are required for photosynthesis as catalysts and bound into complexes, and often sparsely dissolved in aquatic habitats, AAP likely have strong mechanisms to acquire cations for their enhanced needs.

4.6.5. Phylogenetic analysis of AAP and new taxonomic perspectives

Despite the initial steps of 16S rRNA gene sequencing, that revealed each strain related to one of 20 individual type species belonging to several α -proteobacterial families (Table 4.1, Fig. 4.7), we were additionally curious about their specific relatedness. Phylogenetic trees were built to contain most representatives from each nearest neighboring genus, and specifically acknowledge the most related phototrophic species (Fig. 4.8). The *Erythrobacteraceae* have been well characterized as entirely composed of AAP, also supported by our results, since several new isolates were well dispersed throughout the family. In comparison, *Sphingomonadaceae* was predominantly split into *Blastomonas*, well established AAP, and *Sphingomonas*, a genus with both heterotrophs and phototrophs previously identified (Kopejtká *et al.*, 2021). Of note, AJ72 was buried within the branched genus *Sphingorhabdus*, previously undocumented as phototrophic. Since this isolate required specific growth conditions to initiate BChl a synthesis including growth at 28°C, pH 10 and 0-0.1 g/L yeast extract, and has thus far only produced minimal amounts in laboratory settings, perhaps all species of the entire genus physiologically are AAP, and they have yet to be thoroughly investigated. Speaking about *Sphingomonas* relatives, we had two isolates CO70 and BC100 that were related to type species on either end of the genus (Fig. 4.8). We speculate this dispersed photosynthesis provides evidence that the entire genus may have either the capability of photosynthesis, and many species

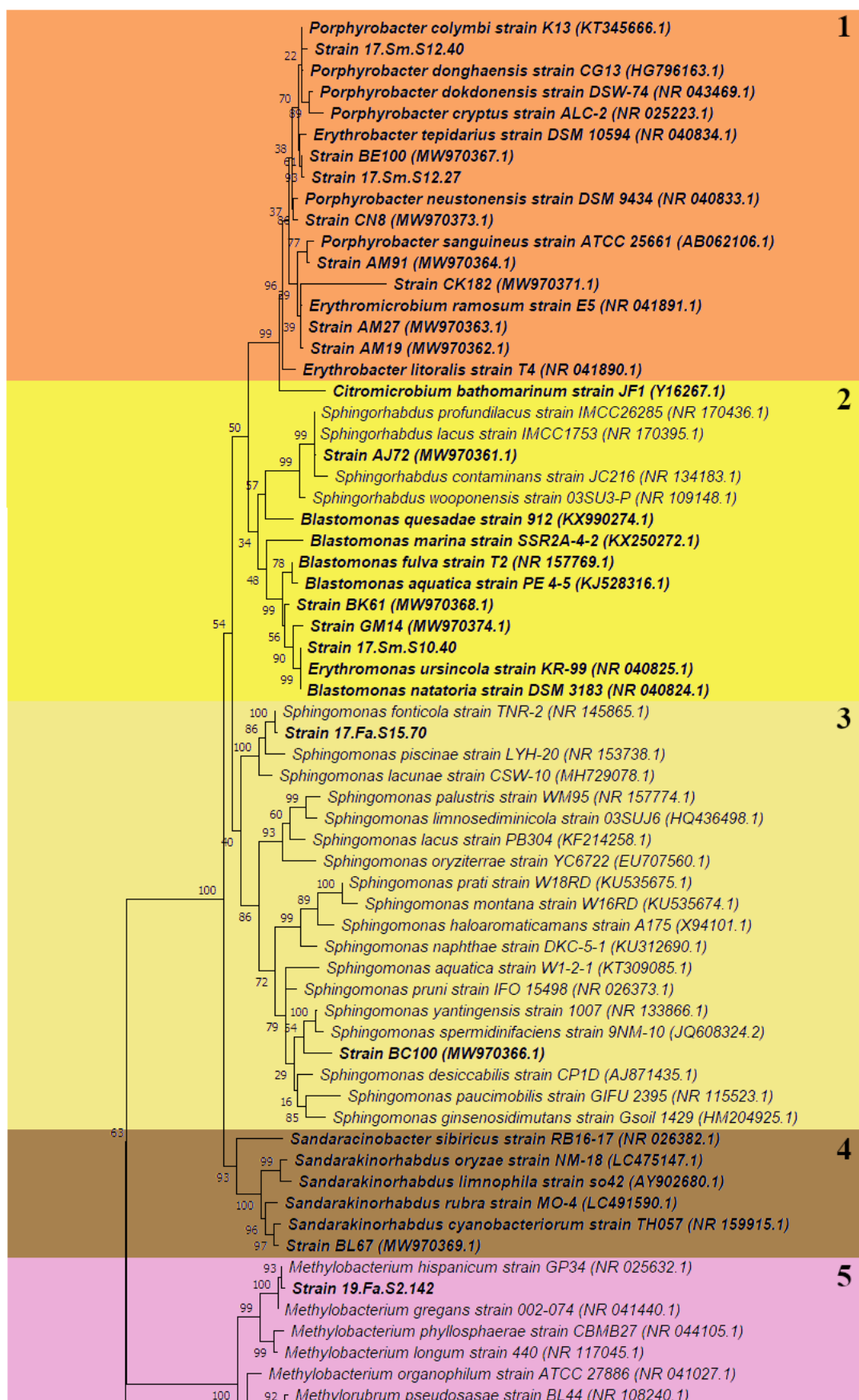


Fig. 4.8. continues next page:



Fig. 4.8. Entire phylogenetic tree of 20 isolated AAP using 16S rRNA gene similarity to all type species of nearest genera, and closest photosynthetic members. Maximum Likelihood method and General Time Reversible model used, with branch length measured by number of substitutions per site. All species identified as AAP are in bold, others not identified as AAP are in slender script. Each family is coloured by generalized carotenoid presentation, and grouped as (1) *Erythrobacteraceae*, (2) *Sphingomonadaceae* of *Blastomonas*/*Sphingorhabdus*/*Citromicrobium* denomination, (3) *Sphingomonadaceae* of *Sphingomonas* lineage, (4) *Sphingosinicellaceae*, (5) *Methylobacteriaceae*, (6) *Rhodobacteraceae*, (7) *Acetobacteraceae*, (8) *Acetobacteraceae*, but of the *Roseomonas* group recently split into 6 genera, (9) β - and γ -*Proteobacterial* AAP, and (10) *Gemmatimonadetes* AAP.

were not grown to detect their pigments, or that the genus now contains heterotrophs without photosynthesis capabilities, as they were lost, but the evolutionary origin of the family started with a phototroph. As these strains also had limited production of BChl *a* under only a few conditions, perhaps several members of *Sphingomonas* that were previously reported without photosynthesis may not have been challenged to the right stimulation for BChl *a* expression. Considering another family, the new strain BL67 related to recent *Sphingosinicellaceae* (Hördt *et al.*, 2020), which is an additional tight group with several characterized aerobic phototrophs. Branching away, four pink AAP isolates CB142, CL63, BA23, and CE55 were spread throughout *Methylobacteriaceae*, indicating at least *Methylobacterium* and *Methylorubrum*, if not the entire family, were likely AAP. Over 2 decades ago, it was reported that some methylophilic bacteria produced BChl *a* (Yurkov and Beatty, 1998), hence we believe they warranted further study as likely representing aerobic phototrophs with unique heterotrophic capabilities. While several members of *Rhodobacteraceae* produced photosynthetic complexes, AC101 related to *Rubellimicrobium* sp., the genus that had only been documented as heterotrophic. Future analysis is necessary here because the low 96% 16S rRNA gene sequence similarity may indicate on a new genus, or may also stimulate reclassification of the related organisms into AAP. *Acetobacteraceae* similarly has had known members of AAP, but here we found strains CK155, CI117, and AE24 diversely spread throughout the genus *Roseomonas*, recently split into 6 new groups that have intermittently been suggested as potentially carrying BChl *a* genes. Our proof of activity strongly speaks that the newly described genera *Neoroseomonas* and *Falsiroseomonas* are likely AAP (Rai *et al.*, 2021), and the type species must be thoroughly tested to validate our hypothesis.

Taken together, *Erythrobacteraceae*, *Sphingomonadaceae* and *Sphingosinicellaceae* members had typical ovoid to rod-shaped cells that were motile, whereas a few elongated forms were not. Likely, this differentiated free pelagic species from those that benefited from larger sizes, potentially weaving into mat-like matrices of cyanobacteria (Kevbrin *et al.*, 2021), or forming associations with filter feeding predators such as zebra mussels (Chapter 3). While *Erythrobacteraceae* had predominantly orange carotenoid composition, *Sphingomonadaceae* and *Sphingosinicellaceae* had similar major yellow presence, where certain additional carotenoids among some members provided a deep brown hue, especially when provided with excess organics. BChl *a* containing RC and LHI complexes were detected most with typical ~867 nm absorbance maximum, with two exceptions made by strains AJ72 and CO70 that had LHI absorbing at 862 nm, relating to *Sphingorhabdus* and *Sphingomonas*, respectively. The *Erythrobacteraceae* filamentous forms also expressed an 835 nm LHII, presumably providing additional light harvesting for the stagnant AAP. While

no *Sphingomonadaceae* strains had LHIII, the *Sphingosinellaceae* related BL67 had a novel complex peak at 855 nm, potentially a feature that might be established in many of its nearest relatives. All three families had similar diverse carbon metabolism and were capable of butyrate and glucose utilization, enzymatic activities, hydrolysis of Tween 60, and resistances to antibiotics such as streptomycin and penicillin G. Stains similar to *E. ramosum* or *B. ursincola* had the highest levels of metalloid oxide tolerance, whereas representatives of distant *Sphingomonas*, *Sphingorhabdus* or *Sandarakinorhabdus* had less. Growth ranges were also similar among *Erythrobacteraceae*, *Sphingomonadaceae* and *Sphingosinellaceae*, with pigments produced constitutively at optimal temperature, and under oligotrophic conditions rather than copiotrophic. The effect of pH did differentiate between these families, where most *Erythrobacteraceae* related strains made BChl *a* in acidity, while all others had no preference for any pH within tolerance. This combination of traits suggested that all AAP of *Sphingomonadales* maintain a wide range of metabolic pathways, have vast tolerance to metals as well as antibiotics, and are capable of active replication in various environments without much limitation. Such attributes are likely explaining why these AAP were the first to be discovered, and are the easiest to isolate from any habitat.

In comparison, the representatives of the three pink-coloured families, *Methylobacteriaceae*, *Acetobacteraceae* and *Rhodobacteraceae* had different attributes. In general, most were collected at littoral locations when grown on OM. This indicated on a preference for low organics, or their capability of surviving starvation, when other community bacteria could not. As these three families belonged to different classes of *Proteobacteria*, they had differential characteristics, as reflected in morphology. *Methylobacteriaceae* isolates CB142, CL63, BA23 and CE55 were pinguis vibroids, while *Acetobacteraceae*, CK155, CI117 and AE24 were coccoid, and *Rhodobacteraceae*, AC101 was moniliform. This delineation was also confirmed by RC and LH absorbance maxima, as they had different LHI with peaks at 869, 854 or 863 nm, respectively. While these LHI shifts were significant, the three families did have similarities as none contained LHIII, all were pink due to the set of carotenoids, polymyxin B resistant, and branched together on the 16S rRNA gene sequence based phylogenetic tree (Fig. 4.7), away from the yellow-orange-brown strains of *Sphingomonadales*. However, these 3 groups evidently had some variation in carotenoid content that made them look as assorted pink hues (Fig. 4.7). The secondary pigments of *Rhodobacteraceae* appeared similar to *Acetobacteraceae* (Fig. 4.2), and these 2 families branched together in the phylogenetic tree (Fig. 4.7). Comparatively the *Methylobacteriaceae* were the only group with a large signal of a putative cytochrome at 361 nm. All members likely require follow-up analysis to determine exact pigment content. Family members

could additionally be separated based on carbon metabolism and enzyme activities, where *Methylobacteriaceae* species maintained the uncommon use of methanol and malate and an inability to produce oxidase (Table 4.2), while both *Acetobacteraceae* and *Rhodobacteraceae* strains had weak catalase activity and no discernable carbon compound supporting their proliferation. While temperature affected all three families similar to *Sphingomonadales*, these pink groups had narrower ranges in pH tolerance, with *Rhodobacteraceae* the smallest. Although *Methylobacteriaceae* and *Rhodobacteraceae* preferred acidic conditions for BChl a production, *Acetobacteraceae* unusually did so in alkalinity. In addition, *Methylobacteriaceae* and *Rhodobacteraceae* isolates grew relatively copiotrophically, whereas *Acetobacteraceae* preferred low organics. However, all produced their primary photosynthesis pigment under nutrient-limited conditions. As *Rhodobacteraceae* branched between *Acetobacteraceae* and *Methylobacteriaceae* via its representatives 16S rRNA gene sequence similarity (Fig. 4-7), it also appeared to share different phenotypic traits between either set of relatives. No representatives of pink families were initially isolated from tellurite enriched medium, which matched their unified intolerance to tellurite (Table 4.4). While high levels of Te tolerance/transformations was a trait of *Sphingomonadales*, selenite could be tolerated and reduced in several *Methylobacteriaceae*, *Rhodobacteraceae*, and *Acetobacteraceae* examples, warranting investigations into enzyme specificity.

Considering all results collectively, several conclusions could be drawn. First, after viewing many AAP examples representing individual species and genera, trends between different families became apparent. Specifically, each had defined characteristics that separated them, while also having common features with their closest genetic relatives. Components of their morphology and physiology were as differential as their pigment compositions and RC and LH absorbance spectra maxima. This was a key point, since it strongly indicated that all were strictly distinguishable from those of other families, and that they had developed many unique traits over the time of steady evolution. In particular, their secondary pigments were strikingly divergent in addition to morphology and key carbon utilization, enzymatic activities or metalloid tolerances. Therefore, such consistency strongly supported the idea that most AAP likely inherited photosynthesis from vertical evolutionary decent, and not from HGT, as also strongly supported in genetic studies of *Methylobacteriaceae* AAP (Zervas *et al.*, 2019). Case in point, cellular carotenoids have been described as partially unlinked to RC and LH complexes, and not entirely reliant for AAP to act photosynthetically (Yurkov *et al.*, 1993). This would indicate that of all components, carotenoids should be the easiest to transfer or loose. However, no single natural *Erythrobacteraceae*, *Sphingomonadaceae*, or *Sphingosinicellaceae* was

pink, and no *Methylobacteriaceae*, *Acetobacteraceae*, or *Rhodobacteraceae* was of yellow-orange hue. Furthermore, LHI and LHII absorption maxima provided observable trends of particular absorbance peaks found only within strains of each family. If recent HGT was more a common event, such trends would not be as clear cut. Thus, each group has contained and maintained their photosynthesis apparatus for an extended period of time to develop these key differences.

Second, this work revealed that definite conditions were required for some AAP to produce BChl a at detectable levels. The slow growth coordinated with enhanced BChl a synthesis suggested that AAP needed specific factors to stimulate photosynthesis, that could be otherwise overlooked in a laboratory setting. Further to this point, our analysis purported that a large diversity of AAP existed in illuminated habitats, including freshwater Lake Winnipeg. The 20 isolates examined here not only matched what was found from environmental sequencing, where genes of *Erythrobacteraceae*, *Sphingomonadaceae*, *Sphingosinicellaceae*, *Methylobacteriaceae*, and *Acetobacteraceae* were identified (Chapter 2), but here we also purified strains related to genera previously undocumented as AAP such as strain AC101 related to *Rubellimicrobium* within *Rhodobacteraceae*, or AJ72 to *Sphingorhabdus* which had not been considered AAP previously. While sequencing *puh/ puf* genes from environmental lake DNA could have identified these species as potential anoxygenic phototrophs, such a culture-independent approach would not have confirmed the aerobic nature of these strains. Since *Rhodobacteraceae* has contained both aerobic and anaerobic species (Pujalte *et al.*, 2014), the identity of related strains from sequencing practices alone would be confounded. This study therefore expands our knowledge of AAP diversity, suggesting that many genera were inappropriately classified in *Proteobacteria* as “strict-heterotrophs” that actually possess a key ecological function, the ability photosynthesize aerobically. With this vision, the reassessment of several type species within these families is necessary, to assay whether or not they can produce BChl a and act as AAP. Currently, many AAP relate to “strict-heterotrophs” or bacteria that do not produce BChl a, particularly in the *Sphingomonas* and *Roseomonas* groups, with suggestion that the photosynthetic function and associated ancestral age, have little importance. However, in light of our findings, we suggest that many of the related “strict-heterotrophs” to our strains may indeed actually be AAP in disguise, awaiting appropriate identification. If this hypothesis is correct, then AAP may be significantly more prevalent among known species, genera, and family of *Proteobacteria*, and make up a more coherent group, indicative of an important physiological role in nature.

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5. Chapter 5.
Discovery of siderophore and metallophore production in the aerobic anoxygenic phototrophs

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5.0. Preamble

The interest in AAP and solving what niche they fill has neither been solely due to their production of BChl a aerobically, nor due to their wide spread nature in most illuminated habitats. Few decades ago, Dr. Yurkov had found a few key members could tolerate high levels of toxic metal(loid) oxides of tellurium, vanadium, and selenium. Such tolerances were made possible from reduction of metal(loid) oxides, and potentially other pathways. This breakthrough, and the additional discovery of a single isolate capable of producing a brown pigment into its surrounding media, led us to ask if AAP could perform several modes of action for metal transformations. Specifically, if any produced siderophores that would bind iron. Taking the idea further, I expanded this concept to see if AAP isolated from numerous sources, including Lake Winnipeg, could produce siderophores for iron, but also metallophores for 9 other different metal(loid)s.

5.1. Contributions statement

Conceptualization, S.B.K., E.H. and V.Y.; methodology, S.B.K., E.H. and V.Y.; software, S.B.K.; validation, S.B.K., V.Y.; formal analysis, S.B.K.; investigation, S.B.K., E.H.; resources, V.Y.; data curation, S.B.K.; writing—original draft preparation, S.B.K., E.H.; writing—review and editing, S.B.K., V.Y.; visualization, S.B.K.; supervision, V.Y.; project administration, V.Y.; funding acquisition, V.Y.

Part of this project was conceptualized with previous graduate students E.H. in V.Y. laboratory, where E.H. initially screened ~20 AAP for siderophores presence and made first attempts to purify siderophore from *C. halotolerans* EG19T as part of her PhD research. Study was expanded by S.B.K. and V.Y. to include 101 AAP strains, and test not only siderophore production, but also 9 additional metallophores. Therefore, all experiments in this Chapter were performed, analyzed, and written by S.B.K.

5.2. Abstract

Aerobic anoxygenic phototrophs have been isolated from a rich variety of environments including marine ecosystems, freshwater and meromictic lakes, hypersaline springs, and biological soil crusts, all in the hopes of understanding their ecological niche. Over 100 isolates were chosen for this study, representing 44 species from 27 genera. Interactions with Fe^{2+} and other metal(loid) cations such as Mg^{2+} , V^{3+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Se^{4+} and Te^{2+} were tested using a chromeazurol S assay to detect siderophore or metallophore production, respectively. Representatives from 20 species in 14 genera of α -Proteobacteria, or 30% of strains, produced highly diffusible siderophores that could bind one or more metal(loid)s, with activity strength as follows: $\text{Fe} > \text{Zn} > \text{V} > \text{Te} > \text{Cu} > \text{Mn} > \text{Mg} > \text{Se} > \text{Ni} > \text{Co}$. In addition, γ -proteobacterial *Chromocurvus halotolerans*, strain EG19 excreted a brown compound into growth medium, which was purified and confirmed to act as a siderophore. It had an approximate size of ~341 Da and drew similarities to the siderophore rhodotorulic acid, a member of the hydroxamate group, previously found only among yeasts. This study is the first to discover siderophore production to be widespread among the aerobic anoxygenic phototrophs, which may be another key method of metal(loid) chelation and potential detoxification within their environments.

Keywords: aerobic anoxygenic phototrophs; siderophore; metallophore; CAS assay; *Chromocurvus halotolerans* strain EG19^T

5.3. Introduction

Iron (Fe) is an essential element for life. In microorganisms, it is used as a co-factor for enzymatic processes, such as in electron transfer during respiration and photosynthesis, nucleic acid or chlorophyll synthesis, nitrate reduction, nitrogen fixation and detoxification of oxygen radicals (Granger *et al.*, 1999; Challis, 2005; Schalk *et al.*, 2011). The use of Fe in bacteriochlorophyll (BChl) synthesis and the process of photophosphorylation makes it particularly important to phototrophic organisms. Aerobic anoxygenic phototrophs (AAP) are one such physiological group that uses photosynthesis in oxic conditions as an additional energy source to respiration (Yurkov and van Gemerden, 1993). They make up a significant proportion of many bacterial communities from a host of environments (Yukov and Hughes, 2017), and therefore likely require a substantial Fe uptake. In addition, Fe may be crucial to protect the cells from oxidative stress due to singlet oxygen formation during BChl a synthesis. While necessary for metabolism, biologically active Fe is typically quite sparse in nature as its soluble level is very low at soil and water surfaces (Challis, 2005). In response to this limitation, both bacteria and fungi have developed siderophores to compete for the available Fe (Faraldo-Gómez and Sansom, 2003). Siderophores, aptly named from the Greek root representing “iron-bearing” (Lankford, 1973), are low weight molecules (no more than 1500–2000 Da and generally lower than 1000 Da) with a high-affinity for Fe (Challis, 2005; Schalk *et al.*, 2011; Reid *et al.*, 1993; Wandersman and Delepelaire, 2004; Grobelak and Hiller, 2017). The molecules are often short polypeptides with modified or D-amino acids (Challis, 2005; Wandersman and Delepelaire, 2004; Crosa and Walsh, 2002). They can also be made from dicarboxylic acids and diamine or amino alcohols, linked by amide and ester bonds. These building blocks retain some characteristics of amino acids (Challis, 2005). Siderophores can be classified into two main functional groups. The first is the hydroxamate group, which involves hydroxamic acid and is produced by both fungi and bacteria. Second is the catechol group, compounds of which contain catechol rings and are only produced by bacteria (Faraldo-Gómez and Sansom, 2003; Wandersman and Delepelaire, 2004; Grobelak and Hiller, 2017). Smaller groups such as the hydroxyacids and the α -hydroxy carboxylates are only rarely used by bacteria (Wandersman and Delepelaire, 2004; Grobelak and Hiller, 2017). Usually, siderophores are synthesized and secreted by cells under iron-deplete growth conditions (Reid *et al.*, 1993). Once bound to iron, the complex is taken up by the cells in a substrate specific process (Challis, 2005).

While the main purpose of siderophores is Fe acquisition, they may also play some additional roles. In *Pseudomonas aeruginosa*, pyoverdine controls virulence factor production (Lamont *et al.*, 2002). *Escherichia*

coli can be protected from oxidative stress by the catechol type siderophore enterobactin (Adler *et al.*, 2012). Pyochelin in *P. aeruginosa* has a toxic effect on eukaryotic cells, possibly aiding in the bacterium's virulence (Adler *et al.*, 2012). Watasemycins, sideromycins, oxachelin and fusigen have antibacterial activity, which may aid in community competition by preventing other populations from growing (Adler *et al.*, 2012). Additionally, siderophores have been shown to bind more than one metal (Schalk *et al.*, 2011; Ghssein *et al.*, 2016), including some that have higher affinity for Cu or Zn rather than Fe (Kim *et al.*, 2004; Morey and Kehl-Fie, 2020). This broad-spectrum activity has required the classification “metallophore” (Welch *et al.*, 1995; Pedler *et al.*, 2000), which is a term used for secondary metabolites capable of binding a range of metal(loid) cations. When a metallophore would have a specific metal affinity, it would have a sub-categorical name, where siderophore is for Fe-binding, chalkophore for Cu, or zincophore for Zn, all named when discovered.

This concept, of metallophores capable of capturing multiple metal cations has implicated usefulness in toxic heavy metal tolerances. Particularly in extreme environments, where metals can be present at elevated concentrations that inhibit a variety of life (Nies, 2000; Rothschild and Mancinelli, 2001). Many metal ions can diffuse freely through the cellular membrane, which is inhibited if the metal is bound to a siderophore that is too large to move without active transport. Furthermore, membrane receptors specific to siderophore-iron complexes can differentiate between those containing substitute cations, causing the cell to reject the alternative metal containing siderophore. The reduction of free metal concentrations in proximity of the bacterium and decreased passive diffusion of unwanted metals into the cell will lower their overall toxic effect (Schalk *et al.*, 2011). This could be a compelling concept, as AAP possess very high levels of tolerance to toxic heavy metal(loid) oxides (Csotonyi *et al.*, 2014). While internal enzymatic reduction takes place (Maltman and Yurkov, 2015), the external production of siderophores may provide an additional layer of defense. Additionally, as mentioned above, the siderophores' ability to reduce reactive oxygen species could help AAP as they need protection against oxidative stress due to their aerobic production of BChl *a*.

Chromocurvus halotolerans EG19^T, is a γ -proteobacterial AAP that was isolated during the spring of 2002 from floating microbial mats within the East German Creek System, Manitoba, Canada (Csotonyi *et al.*, 2008). As this is a landlocked hypersaline spring system, it likely contains highly endemic communities of microorganisms that have not been mixed with or affected by allochthonous populations (Csotonyi *et al.*, 2008). EG19^T forms motile, short rod or longer curved rodshaped, orange-pink bacteria. When grown with

complex carbon sources, EG19^T produces a brown pigmented hydrophilic compound, which is excreted into the growth medium. While a similar phenomenon had never been reported in other AAP, it was hypothesized that the compound could be a siderophore (Csotonyi *et al.*, 2011), as ferric bound siderophores can be visually yellow-brown or red-brown (Drechsel and Jung, 1998). Our study confirms the identity of this extracellular product and describes it as the first siderophore discovered in an AAP. Other AAP from a vast array of environments that do not pigment growth medium were also tested for their production of siderophores, as most of these metal chelating small molecules are colourless, and synthesis is therefore possible.

5.4. Material and Methods

5.4.1. Bacterial strains and growth conditions

For this study, 101 strains of AAP originating from an assortment of environments including Lake Winnipeg, as well as phylogenetically diverse throughout numerous proteobacterial clades, were selected from Dr. Vladimir Yurkov's vast collection. A complete list of chosen strains, original source of isolation, relatedness to type species, and 16S rRNA partial gene sequence accession numbers are listed in Tables 5.1 and 5.2.

Freshwater AAP were cultivated on rich organic (RO) medium as described (Yurkov, 2006), with one minor modification. Bactopectone was reduced to 0.5 g/L and casamino acids were supplemented at 0.5 g/L, which provided a larger variety of complex nutrients. Marine AAP requiring salt were propagated on RO medium described above supplemented with 2% NaCl. AAP originally isolated from the East German Creek System, Manitoba, Canada, were grown using medium A (MA) (Csotonyi *et al.*, 2008). Those isolated from biological soil crusts of Sandy Lands Forest and Spruce Woods National Park were cultured on Biological Soil Crust Medium A or B (BSCA or BSCB) (Csotonyi *et al.*, 2010). Strains from meromictic Mahoney Lake in British Columbia, Canada were grown on N1 medium (Yurkova *et al.*, 2002).

In addition to bacterial isolates formerly described elsewhere, some of those chosen for siderophore testing had not been previously published. AAP isolated from the meromictic Blue Lake in British Columbia, Canada, were cultured using a Blue Lake medium (BLM) containing (g/L): MgSO₄, 0.5; NH₄Cl, 0.3; KH₂PO₄, 0.3; KCl, 0.3; CaCl₂, 0.05; NaCl, 12.0; NaHCO₃, 0.5; Na-acetate, 1.0; malic acid, 1.0; yeast extract, 1.0; bactopectone, 0.5; with vitamins and trace elements solutions, 2.0 mL each; autoclaved at pH 5.9 and then adjusted after autoclaving to pH 7.5 with 0.5 N NaOH.

Table 5.1. Freshwater and saline AAP chosen for siderophore testing.

Environment	Strain	Medium	Most related type species	Accession #
Hot spring, Kamchatka island	KR99 ^T	RO	<i>Erythromonas ursincola</i>	NR_119243.1
Warm temperature spring, Baikal Lake, Russia	E1	RO	99.6% <i>Porphyrobacter colymbi</i>	MW970346
	E4(1)	RO	99.7% <i>Porphyrobacter donghaensis</i>	MW970347
	E5 ^T	RO	<i>Erythromicrobium ramosum</i>	NR_041891.1
	RB3 ^T	RO	<i>Roseococcus thiosulfatophilus</i>	NR_026114.1
	RB16-17 ^T	RO	<i>Sandaracinobacter sibiricus</i>	NR_026382.1
	T4 ^T	RO - NaCl	<i>Erythrobacter litoralis</i>	NR_119016.1
Deep Ocean, Juan De Fuca Ridge, Pacific Ocean	JF1 ^T	RO - NaCl	<i>Citromicrobium bathyomarinum</i>	Y16267.1
	C6	RO - NaCl	98.1% <i>Citromicrobium bathyomarinum</i>	MW970348
	C7	RO - NaCl	99.8% <i>Citromicrobium bathyomarinum</i>	MW970349
	C8	RO - NaCl	99.9% <i>Citromicrobium bathyomarinum</i>	MW970350
	C14	RO - NaCl	99.6% <i>Citromicrobium bathyomarinum</i>	MW970351
	C23	RO - NaCl	99.7% <i>Citromicrobium bathyomarinum</i>	MW970352
	C26	RO - NaCl	99.8% <i>Citromicrobium bathyomarinum</i>	MW970353
	N25	RO - NaCl	99.7% <i>Citromicrobium bathyomarinum</i>	MW970354
	N34	RO - NaCl	99.7% <i>Citromicrobium bathyomarinum</i>	MW970355
	N48	RO - NaCl	99.8% <i>Citromicrobium bathyomarinum</i>	MW970356
	N56	RO - NaCl	99.9% <i>Citromicrobium bathyomarinum</i>	MW970357
Rag Beach sediment, BC, Canada	N78	RO - NaCl	99.6% <i>Citromicrobium bathyomarinum</i>	MW970358
	15-SB	RO - NaCl	99.8% <i>Porphyrobacter donghaensis</i>	MW970359
	23-SB	RO - NaCl	99.7% <i>Porphyrobacter donghaensis</i>	MW970360
Central Gold Mine, MB, Canada	C4	RO	99.4% <i>Porphyrobacter colymbi</i>	KX148515
	C9	RO	99.1% <i>Brevundimonas variabilis</i>	KX148516
	C11	RO	98.6% <i>Brevundimonas bacterioides</i>	KX148517
	NM4.16	RO	99.7% <i>Porphyrobacter colymbi</i>	KX148518
	NM4.18	RO	99.3% <i>Blastomonas fulva</i>	KX148519
Lake Winnipeg, MB, Canada	AJ 72	RO	99.7% <i>Sphingorhabdus lacus</i>	MW970361
	AM 19	RO	99.7% <i>Erythromicrobium ramosum</i>	MW970362
	AM 27	RO	99.6% <i>Erythromicrobium ramosum</i>	MW970363
	AM 91	RO	98.5% <i>Porphyrobacter sanguineus</i>	MW970364
	BA 23	RO	96.2% <i>Methylobacterium indicum</i>	MW970365
	BC 100	RO	96.6% <i>Sphingomonas yantingensis</i>	MW970366
	BE 100	RO	99.4% <i>Porphyrobacter colymbi</i>	MW970367
	BK 61	RO	99.1% <i>Blastomonas fulva</i>	MW970368
	BL 67	RO	98.9% <i>Sandarakinorhabdus cyanobacteriorum</i>	MW970369
	CK 155	RO	98.1% <i>Roseomonas sediminicola</i>	MW970370
	CK 182	RO	96.2% <i>Porphyrobacter donghaensis</i>	MW970371
	CL 63	RO	100% <i>Methylobacterium extorquens</i>	MW970372
	CN 8	RO	99.2% <i>Porphyrobacter neustonensis</i>	MW970373
	GM14	RO	99.2% <i>Erythromonas ursincola</i>	MW970374
Zebra mussels, Lake Winnipeg, MB, Canada	Z1	RO	99.1% <i>Porphyrobacter colymbi</i>	MN987006
	Z2	RO	99.4% <i>Porphyrobacter tepidarius</i>	MN987007
	Z5	RO	99.6% <i>Porphyrobacter tepidarius</i>	MN987008
	Z6	RO	99.3% <i>Porphyrobacter neustonensis</i>	MN987009
	Z7	RO	99.6% <i>Erythromicrobium ramosum</i>	MN987010
	Z24	RO	98.7% <i>Porphyrobacter sanguineus</i>	MN987011
	Z27	RO	99.4% <i>Porphyrobacter neustonensis</i>	MN987012
	Z39	RO	98.9% <i>Porphyrobacter sanguineus</i>	MN987013
	Z59	RO	99.5% <i>Porphyrobacter neustonensis</i>	MN987014
	Z68	RO	99.4% <i>Porphyrobacter neustonensis</i>	MN987015

Table 5.2. Meromictic lake and biological soil crust AAP chosen for siderophore testing.

Environment	Strain	Medium	Most related type species	Accession #
Mahoney Lake, BC, Canada	ML1	N1	98.4% <i>Erythromicrobium ramosum</i>	MW970375
	ML3	N1	99.4% <i>Porphyrobacter sanguineus</i>	MW970376
	ML4 ^T	N1	<i>Porphyrobacter meromictius</i>	NR 115007.1
	ML6 ^T	N1	<i>Roseicyclus mahoneyensis</i>	NR 042080.1
	ML10	N1	97.9% <i>Salinarimonas ramus</i>	MW970377
	ML14	N1	98.9% <i>Erythromicrobium ramosum</i>	MW970378
	ML19	N1	96.9% <i>Porphyrobacter meromicticus</i>	MW970379
	ML20	N1	99.3% <i>Blastomonas fulva</i>	MW970380
	ML21	N1	98.5% <i>Erythromicrobium ramosum</i>	MW970381
	ML22	N1	99.8% <i>Porphyrobacter sanguineus</i>	MW970382
	ML30	N1	98.8% <i>Seohaecicola saemankumensis</i>	MW970383
	ML35	N1	99.4% <i>Blastomonas fulva</i>	MW970384
	ML36	N1	97.1% <i>Porphyrobacter sanguineus</i>	MW970385
	ML37	N1	95.4% <i>Glycocalyx profundus</i>	MW970386
	ML42	N1	98.0% <i>Roseicyclus marinus</i>	MW970387
	ML45	N1	94.7% <i>Ruegeria intermedia</i>	MW970388
Blue Lake, BC, Canada	BL1	BLM	98.1% <i>Roseicyclus marinus</i>	MW970389
	BL4	BLM	99.3% <i>Porphyrobacter sanguineus</i>	MW970390
	BL5	BLM	99.2% <i>Seohaecicola saemankumensis</i>	MW970391
	BL7	BLM	95.6% <i>Glycocalyx profundus</i>	MW970392
	BL8	BLM	98.1% <i>Roseicyclus marinus</i>	MW970393
	BL9	BLM	98.0% <i>Roseicyclus marinus</i>	MW970394
	BL11	BLM	95.8% <i>Glycocalyx profundus</i>	MW970395
	BL14	BLM	95.6% <i>Glycocalyx profundus</i>	MW970396
	BL17	BLM	95.1% <i>Glycocalyx profundus</i>	MW970397
	BL22	BLM	95.0% <i>Glycocalyx profundus</i>	MW970398
East German Creek System, MB, Canada	EG1	MA	95.2% <i>Roseovarius pacificus</i>	AM691094
	EG2	MA	94.4% <i>Roseovarius bejariae</i>	AM691093
	EG3	MA	97.3% <i>Yoonia vestfoldensis</i>	AM691092
	EG4	MA	98.8% <i>Erythrobacter longus</i>	AM691105
	EG5	MA	95.7% <i>Roseovarius pacificus</i>	AM691095
	EG6	MA	98.4% <i>Porphyrobacter meromictius</i>	AM691106
	EG7	MA	97.3% <i>Roseovarius nitratreducens</i>	AM691097
	EG8	MA	99.0% <i>Roseovarius tolerans</i>	AM691101
	EG9	MA	97.3% <i>Roseovarius nitratreducens</i>	AM691098
	EG10	MA	98.2% <i>Roseovarius tibetensis</i>	AM691100
	EG11	MA	97.7% <i>Roseovarius nitratreducens</i>	AM691099
Sandy Lands Forest soil crust, MB, Canada	EG13	MA	99.0% <i>Roseovarius tolerans</i>	AM691102
	EG15	MA	98.9% <i>Erythrobacter aquimaris</i>	AM691107
	EG17 ^T	MA	<i>Charonmicrobium ambiphototrophicum</i>	AM691091
	EG19 ^T	MA	<i>Chromococcus halotolerans</i>	AM691088
	SS56	BSCA	99.6% <i>Methylobacterium brachiatum</i>	MW970399
	SS63	BSCA	99.1% <i>Bosea lupini</i>	MW970400
	SS335	BSCA	98.3% <i>Bosea lupini</i>	MW970401
Spruce Woods National Park soil crust, MB, Canada	J01	BSCA	98.4% <i>Belnapia moabensis</i>	MW970402
	J05	BSCA	96.6% <i>Sphingomonas pruni</i>	MW970403
	P4	BSCB	99.6% <i>Methylobacterium phyllosphaerae</i>	MW970404
	P13	BSCB	98.7% <i>Bosea lupini</i>	MW970405
	P40	BSCB	99.9% <i>Belnapia soli</i>	MW970406
	P132	BSCB	99.6% <i>Methylobacterium brachiatum</i>	MW970407
	P233	BSCB	99.8% <i>Methylobacterium tardum</i>	MW970408

Furthermore, several AAP had been recovered from the freshwaters of Lake Winnipeg whilst the habitat was under study (Chapters 1-3). Specifically, in the spring of 2017, strain AJ72 was collected at Grand Beach from littoral water, AM19, AM27 and AM91 were from littoral sediment of Victoria Beach. During that summer, BA23 and BE100 were isolated at limnetic sites S1 and S5, respectively, while BL67 and BK61 originated within the littoral water and sediment of Grand Beach and Victoria Beach, respectively. Fall samples of Grand Beach sediment contained CK155 and CK182, while Victoria Beach littoral waters revealed isolate CL63. All were purified on the slightly modified freshwater RO medium as described.

5.4.2. *Iron chelating chromeazurol S assay*

Every chosen strain was grown on 2% agar plates containing their specific growth medium supplemented with the dye chromeazurol S (CAS), which turns blue when bound to Fe and reverts to yellow/orange being released (Schwyn and Neilands, 1987). To make media, 60.5 mg CAS were dissolved in 50 mL ddH₂O, then mixed with 10 mL of an iron solution containing 1 mM FeCl₃ and 10 mM HCl. HDTMA (72.9 mg) was dissolved in 40 mL ddH₂O prior to mixing with the CAS/iron solution, bringing the total volume to 100 mL. Separately, 900 mL of each growth medium (MA, RO, RO 2% NaCl, BSCA, or BLM) were prepared without the addition of iron, but with the correct amount of components for 1 L, as that would be the final volume of medium after combining with CAS/iron solution. Media were then autoclaved at pH 5.9, separate from the CAS mixture. After autoclaving, each medium was adjusted to pH 6.8 and the CAS solution was added. Agar plates would turn blue when solidified. If measurements of ingredients were not exact or added in an incorrect order, CAS would precipitate out of solution and plates with pH higher than 6.8 would appear green instead of blue. CAS plates were heavily inoculated with each strain, and streaked to achieve isolated colonies after 5 days of growth at 28 °C in the dark.

5.4.3. *Variant cation chromeazurol S assay*

Due to the principle chemistry behind the siderophore assay (Schwyn and Neilands, 1987), which used negatively charged CAS dye that would weakly bind the cation Fe²⁺, we decided to replace Fe²⁺ with different metal(loid) cations, including Mg²⁺, V³⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Se⁴⁺ and Te²⁺. Microbial growth with an aura of colour change in medium from blue to yellow hue would signify that microbially produced siderophore could bind the substituted cation, causing the released dye to revert to its yellow colour. These variant cation CAS plates were successfully created as explained in the previous section, cured to diverse shades of blue dependent on the metal(loid), and were screened for metallophore production identically as

in the Fe-chelation CAS assay. All cations were purchased from Sigma Aldrich, USA as chloride salts, which were soluble once acidified as described.

5.4.4. Phylogeny

To assess the phylogenetic position, 16S rRNA gene sequences were either acquired from previously published sources, or newly sequenced in this study. A list of accession numbers to partial 16S rRNA gene sequences attained from public repositories was provided in Tables 5.1 and 5.2. From unsequenced strains, DNA was extracted via the phenol chloroform method (Rainey *et al.*, 1996), and sequenced using Sanger technique (Sanger and Coulson, 1975). Primer set 27F and 1492R, 5' -AGAGTTTGATCCTGGCTCAG-3' and 5' -GGTACCTTGTTACGACTT-3', respectively, was used to achieve a total contiguous 16S rRNA gene sequence length of >1400 bp per strain. New sequences of 16S rRNA genes from previously unidentified AAP strains were deposited to GenBank under accession numbers (MW970346–MW970408) as listed in Tables 5.1 and 5.2. Genetic relation of 16S rRNA gene sequences acquired from each AAP isolate were compared to the archived sequences of type species using the web-based software Basic Local Alignment Search Tool, BLAST (Madden, 2002). Phylogenetic trees were constructed via MEGA X software (Kumar *et al.*, 2018) with 1000 bootstraps (Felsenstein, 1985), using Maximum Likelihood method to align all AAP 16S rRNA gene sequences to one another based on the General Time Reversible model (Nei and Kumar, 2000). As AAP from across proteobacteria were tested, *Deinococcus* was used as an outgroup from a separate phylum. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

5.4.5. Siderophore isolation and concentration from *C. halotolerans*

Strain EG19^T was grown at 28 °C on MA agarized (2%) plates in the dark for 5 days. Pink-purple colonies developed with brown hue dispersed in agarized medium (Fig. 5.1A), which corresponded to zone of clearing on Fe-Chromeazurol S Assay (Fig. 5.1B). To obtain liquid cultures, EG19^T was inoculated at 5% in Fe-free MA and grown for 5 days at 28 °C shaking at 200 rpm in the dark. Fe-free MA was prepared as described in chromeazurol S assay, excluding Fe from the trace element solution. In addition, after all components were added, 5 g/L of Chelex resin enclosed in dialyzing tubing was placed in the medium for 1 h prior to autoclaving. This step allowed the Chelex resin to remove traces of Fe introduced through the complex nutrients such as yeast extract, bactopectone and casamino acids (Barker *et al.*, 1979).

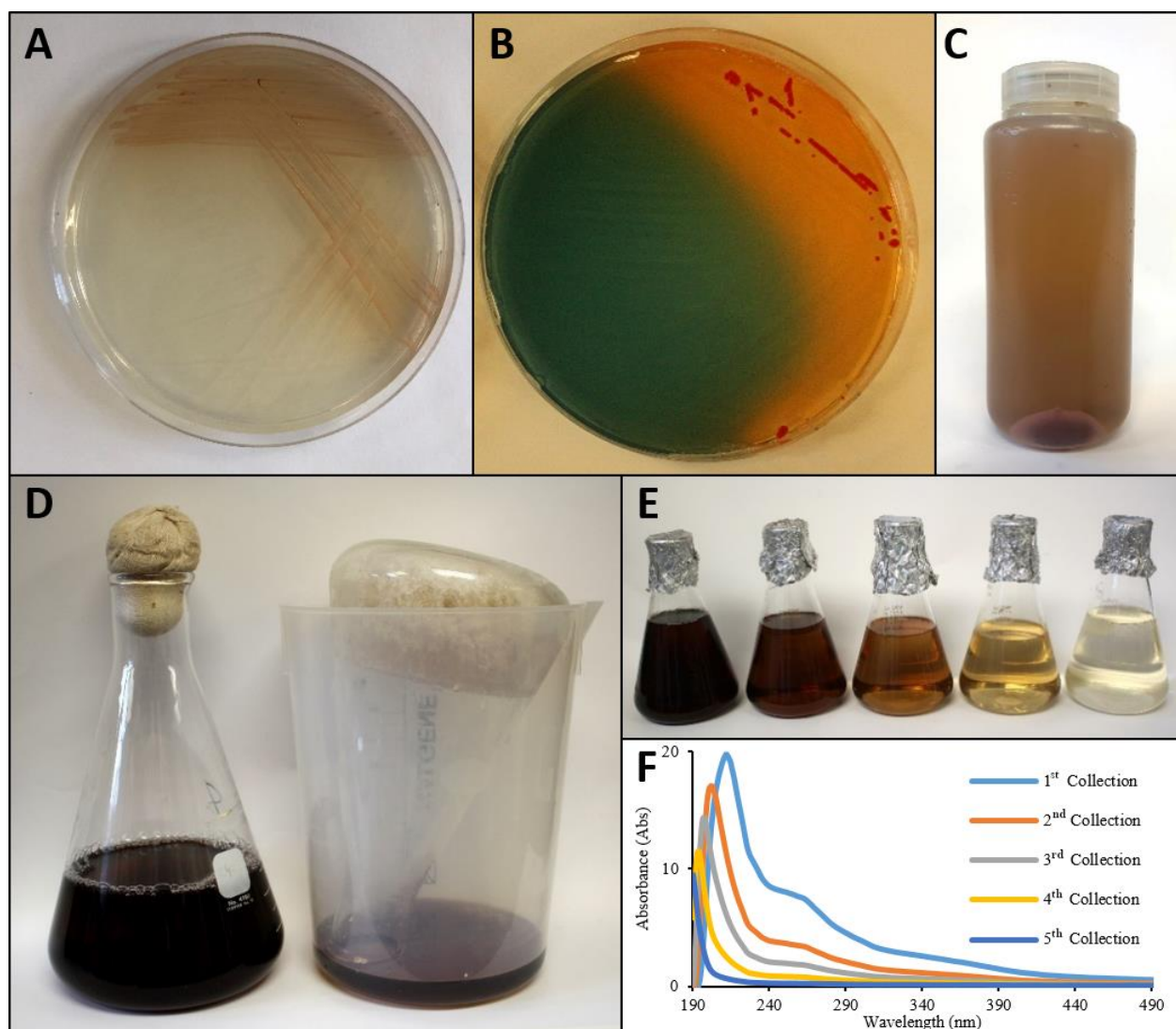


Fig. 5.1. Extraction and concentration of brown pigment excreted by *C. halotolerans*. (A) Strain EG19^T released a brown pigment into MA medium. (B) Fe-CAS plate with siderophore production by EG19^T. (C) Pelleted cells under supernatant. (D) Pigment concentrated with freezing-out technique. (E) Collected fractions during thawing with concentrated pigment released first. (F) Absorbance spectrum of each fraction.

After the cultivation of *C. halotolerans*, Fe-free medium became dark brown. The pink-purple cells were pelleted in ~450 mL bottles at 10,000 rpm for 30 min using a Beckman J2HS centrifuge and a JA-10 rotor (Fig. 5.1C). Siderophore containing supernatant was collected and 1.25 L was transferred into 2 L Erlenmeyer flasks (Fig. 5.1D) to freeze overnight at -20°C . Highly concentrated dark brown-pigmented high-salt slurry was formed predominantly on top of ice, while the lower part of ice was close to colourless. The flask with frozen material was removed from freezer and allowed to defrost at room temperature inserted upside down in a new collection beaker. Thawed supernatant was fractioned into 250 mL batches, with concentrated siderophore thawed and collected first, leaving frozen medium behind (Fig. 5.1D), both observable visually (Fig. 5.1E), and from the absorbance spectrum (Fig. 5.1F).

A batch-type method of siderophore purification was used in this study (Yamamoto *et al.*, 1994). Here, the combined freeze-concentrated samples were adjusted to pH 6.0 and XAD 7-HP resin was added (20 g resin per L of supernatant). This slurry was shaken at 200 rpm for 1 h on a rotary shaker and then filtered through a glass Millipore filter funnel that would collect resin, but allow supernatant to easily flow through. Concentrated siderophore required several extractions with resin to remove all pigment from supernatant. Siderophore-bound-resin (now brown in colour) was thoroughly washed with ddH₂O to remove all residual salts and other soluble contaminants. Vacuum assisted drying using Millipore system was performed, ddH₂O discarded, and the cleaned siderophore-bound-resin was soaked in methanol for 30 min to release the siderophore. Pigmented methanol was collected, and resin was soaked once more using the same batch of solvent for additional extraction. Methanol extracts were combined and then evaporated to dryness. The resulting dark brown powder was the presumed highly concentrated siderophore, and kept at -20°C until further testing. Unbound-resin was washed with methanol, then soaked with ddH₂O prior to reuse.

5.4.6. Purification and Fe-chelation of siderophore from *C. halotolerans*

Concentrated siderophore powder from resin purification was resuspended in ddH₂O (10 mg in 50 μL), and decimally diluted up to 10^{-3} . In addition, a solution containing 5 mg of concentrate in 100 μL ddH₂O was filtered through an Amicon® Ultra 0.5 mL 3000 Da spin column manufactured by Millipore Ireland, which removed any contaminating proteins over that size. This filtered concentrate was also decimally diluted up to 10^{-2} . Once the dilutions were made, 10 μL of each sample, and an aliquot of freeze-thawed concentrate, were individually mixed with 10 μL of loading buffer, prior to filling into the wells of a Mini-Protean tris-tricine gel, 16.5% from Bio-Rad, Hercules, CA, USA. Loading buffer contained 200 mM TRIS-HCl, pH 6.8,

2% SDS, 40% glycerol and 0.04% Coomassie Brilliant Blue G-250 (CBB) from Bio-Rad, USA. Running buffer was made up of 1 M tris, 1 M tricine and 1% SDS at pH 8.3. Wells were 30 μ L, filled with 20 μ L of sample (10 μ L of sample, 10 μ L of loading buffer). Protein ladder was a C6210—color marker ultra-low range (M.W. 1,060–26,600) from Sigma-Aldrich, St. Louis, MO, USA. Gel electrophoresis was run at 100 volts for 1 h prior to being stained in a CBB solution (1 g of CBB dissolved in 1 L of [50% MeOH, 10% glacial acetic acid, 40% H₂O]). The gel was then destained with a mixture containing 5% acetic acid and 20% MeOH overnight. Fe chelation was tested with the concentrated siderophore powder from the resin concentration step, the brown pigment filter-purified below 3 kDa, as well as with a sample from the remaining proteins that were >3 kDa from the spin filtration procedure. Each fraction was solubilized, where 10 mg of dried pigment was dissolved in 500 μ L of 60% MeOH, prior to applying each solution into a blank diffusion disk, manufactured by Oxoid in the UK, allowing it to become dry and concentrated within the disk. These were then placed on a Fe-CAS plate and left to react with chromeazurol S overnight.

5.5. Results

5.5.1. Bacterial growth and Fe-CAS-plate reactions

Comparative phenotypic analysis was achieved by allowing each strain to develop for 5 days on their respective agarized media under conditions that promoted the best growth. This elapsed time ensured stationary phase was reached for each representative, which resulted in the formation of sufficient colonies for analysis. Triplicate CAS-supplemented and CAS-free controls were simultaneously plated to identify the viability of inoculum. In all cases, growth occurred on CAS plates, but was marginally reduced in comparison to controls. Both after 3 and 5 days, the average zone of colour change (blue to yellow) was recorded, revealing several phenotypic attributes. As some strains grew slower than others, 5 days' period was chosen to analyze and compare all simultaneously. We identified siderophore production based on zone of diffusion/colour change around colonies as follows (Fig. 5.3): no zone (–), a zone <1 mm around colonies (+), a moderate diffusion <10 mm (++), and considerable diffusion >10 mm (+++). Fe-chelating CAS reactions after 5 days are listed in Tables 5.3 and 5.4, as well as shown in Fig. 5.2 beside each strain name.

5.5.2. Substitute Cation CAS Assays

Once Fe-chelating siderophore production was confirmed using the standard Fe-CAS assay, all strains were tested on CAS supplemented agar plates that contained one of 9 other metal(loid) cations: Mg²⁺, V³⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Se⁴⁺ and Te²⁺. Results for varied CAS assays are listed along-side Fe²⁺ data in

Table 5.3. Freshwater and saline AAP analyzed for metal(loid)-chelation via CAS assays with varied cations.

Environment	Strain	Medium	Mg	V	Mn	Fe	Co	Ni	Cu	Zn	Se	Te
Hot spring, Kamchatka Isl.	KR99 ^T	RO	++	+++	++	+++	++	++	++	+++	++	+++
Warm temperature spring, Baikal Lake, Russia	E1	RO	-	-	+	++	+	+	-	-	-	-
	E4(1)	RO	-	-	-	++	-	-	-	-	-	+
	E5 ^T	RO	-	-	-	+	-	-	-	-	-	+
	RB3 ^T	RO	-	-	++	++	-	++	+	+	+	+
	RB16-17 ^T	RO	-	-	-	+	-	-	+	-	-	-
	T4 ^T	RO - NaCl	+	+	+	-	+	+	-	-	+	+
Deep Ocean, Juan De Fuca Ridge, Pacific Ocean	JF1 ^T	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	C6	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	C7	RO - NaCl	+	+	+	+	-	+	+	+	+	+
	C8	RO - NaCl	+	+	+	+	-	+	+	+	+	+
	C14	RO - NaCl	+	+	+	+	-	+	+	+	+	+
	C23	RO - NaCl	-	+	+	+	+	+	+	+	+	+
	C26	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	N25	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	N34	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	N48	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	N56	RO - NaCl	+	+	+	+	+	+	+	+	+	+
	N78	RO - NaCl	+	+	+	+	+	+	+	+	+	+
Rag Beach sediment, BC, Canada	15-SB	RO - NaCl	+	-	+	+	-	-	+	+	+	+
	23-SB	RO - NaCl	+	-	+	+	+	+	-	+	-	-
Central Gold Mine, MB, Canada	C4	RO	-	+	+	+	+	+	+	+	-	+
	C9	RO	-	+	-	+	+	-	+	+	-	+
	C11	RO	-	+	-	+	-	-	+	+	-	-
	NM4.16	RO	-	+	+	++	+	+	+	+	-	+
	NM4.18	RO	++	+	+	++	++	++	++	+	++	++
Lake Winnipeg, MB, Canada	AJ 72	RO	-	+	+	+++	+	+	+	++	-	+
	AM 19	RO	-	+	-	+++	-	-	-	++	-	-
	AM 27	RO	-	-	-	++	-	-	-	+	-	-
	AM 91	RO	-	-	-	-	-	-	-	-	-	-
	BA 23	RO	+	++	++	++	++	+	++	++	++	++
	BC 100	RO	-	-	-	++	+	-	-	-	-	-
	BE 100	RO	++	++	++	+++	++	++	++	++	++	+
	BK 61	RO	+	+	+	+++	+	-	+	++	+	-
	BL 67	RO	-	-	-	-	+	-	-	-	-	-
	CK 155	RO	-	-	-	++	-	-	-	-	-	-
	CK 182	RO	-	-	-	-	-	-	-	-	-	-
	CL 63	RO	+	++	++	++	++	++	++	++	++	++
	CN8	RO	+	++	+	++	+	+	-	++	++	+
	GM14	RO	++	++	++	+++	++	+	-	++	++	+
Zebra mussels, Lake Winnipeg, MB, Canada	Z1	RO	-	-	-	+	-	-	-	-	-	-
	Z2	RO	-	-	-	-	-	-	-	-	-	-
	Z5	RO	-	-	-	-	-	-	-	-	-	-
	Z6	RO	+	-	+	-	+	+	-	-	+	-
	Z7	RO	+	-	-	-	-	-	-	-	-	-
	Z24	RO	-	-	-	++	-	-	-	+	-	-
	Z27	RO	+	+	+	++	-	-	-	++	+	++
	Z39	RO	-	-	+	++	+	-	-	+	-	-
	Z59	RO	+	-	+	+	-	+	-	-	+	-
	Z68	RO	-	++	-	++	-	+	-	++	-	+

Table 5.4. Meromictic lake, saline spring and biological soil crust AAP analyzed for metal(oid)-chelation via CAS assays with varied cations.

Environment	Strain	Medium	Mg	V	Mn	Fe	Co	Ni	Cu	Zn	Se	Te
Mahoney Lake, BC, Canada	ML1	N1	-	+	-	+	-	-	+	+	-	+
	ML3	N1	-	+	-	+	-	-	+	+	-	+
	ML4 ^T	N1	-	+	-	+	-	-	+	+	-	+
	ML6 ^T	N1	+	+	+	+	-	-	+	+	-	+
	ML10	N1	-	+	-	+	-	-	+	+	-	+
	ML14	N1	-	+	-	+	-	-	+	+	-	+
	ML19	N1	+	+	+	+	+	-	+	+	+	+
	ML20	N1	-	+	-	+	-	-	+	+	-	+
	ML21	N1	-	+	-	+	-	-	+	+	-	+
	ML22	N1	-	+	+	+	-	-	+	+	-	+
	ML30	N1	+	+	-	+	-	-	+	+	+	+
	ML35	N1	-	+	-	+	-	-	+	+	-	+
	ML36	N1	+	+	+	+	+	-	+	+	+	+
	ML37	N1	-	+	+	+	-	-	+	+	-	+
	ML42	N1	+	+	+	+	+	+	+	+	+	+
	ML45	N1	+	+	+	+	-	+	+	+	+	+
Blue Lake, BC, Canada	BL1	BLM	+	+	+	+	+	+	+	+	+	+
	BL4	BLM	-	+	-	+	-	-	+	+	-	+
	BL5	BLM	+	+	+	+	+	+	+	+	+	+
	BL7	BLM	+	+	+	-	+	+	+	-	+	+
	BL8	BLM	+	+	+	+	+	+	+	+	+	+
	BL9	BLM	+	+	+	+	+	+	+	+	+	+
	BL11	BLM	+	+	+	-	+	+	+	+	+	+
	BL14	BLM	+	+	+	-	+	+	+	+	+	+
	BL17	BLM	+	+	+	+	+	+	+	+	+	+
	BL22	BLM	+	-	+	-	+	+	+	-	+	+
East German Creek System, MB, Canada	EG1	MA	-	+	-	+	-	-	+	+	-	+
	EG2	MA	+	+	+	+	-	-	+	+	-	+
	EG3	MA	-	-	-	+	-	-	-	-	-	-
	EG4	MA	-	+	-	+	-	-	+	+	-	+
	EG5	MA	-	+	+	+	-	-	+	+	-	+
	EG6	MA	-	+	-	+	-	-	+	+	-	+
	EG7	MA	-	+	+	+	-	-	+	+	-	+
	EG8	MA	-	+	-	+	+	-	+	+	+	+
	EG9	MA	-	+	-	+	-	-	+	+	-	+
	EG10	MA	-	+	-	+	-	-	+	+	+	+
	EG11	MA	-	+	-	+	-	-	+	+	-	+
	EG13	MA	-	+	-	+	-	-	+	+	-	+
	EG15	MA	-	+	-	+	+	-	+	+	-	+
	EG17 ^T	MA	+	+	+	+	+	+	+	+	-	+
	EG19 ^T	MA	+	+	+	++	-	+	+	++	+	+
Sandy Lands Forest soil crust, MB, Canada	SS56	BSCA	++	++	++	+	++	++	++	++	++	++
	SS63	BSCA	++	+++	++	+++	++	+++	+++	+++	+++	+++
	SS335	BSCA	+++	+++	+++	+++	++	++	+++	++	++	+++
Spruce Woods National Park soil crust, MB, Canada	J01	BSCA	+++	++	+++	+++	+	++	++	++	+	++
	J05	BSCA	+	+	+	++	-	-	+	+	-	+
	P4	BSCB	++	++	++	+	+	+	++	++	++	++
	P13	BSCB	+++	+++	+++	+++	++	+++	+++	+++	++	++
	P40	BSCB	++	++	++	++	+	++	++	++	+	+
	P132	BSCB	++	++	++	+++	++	++	++	++	++	++
	P233	BSCB	-	++	++	+++	++	++	++	++	++	++

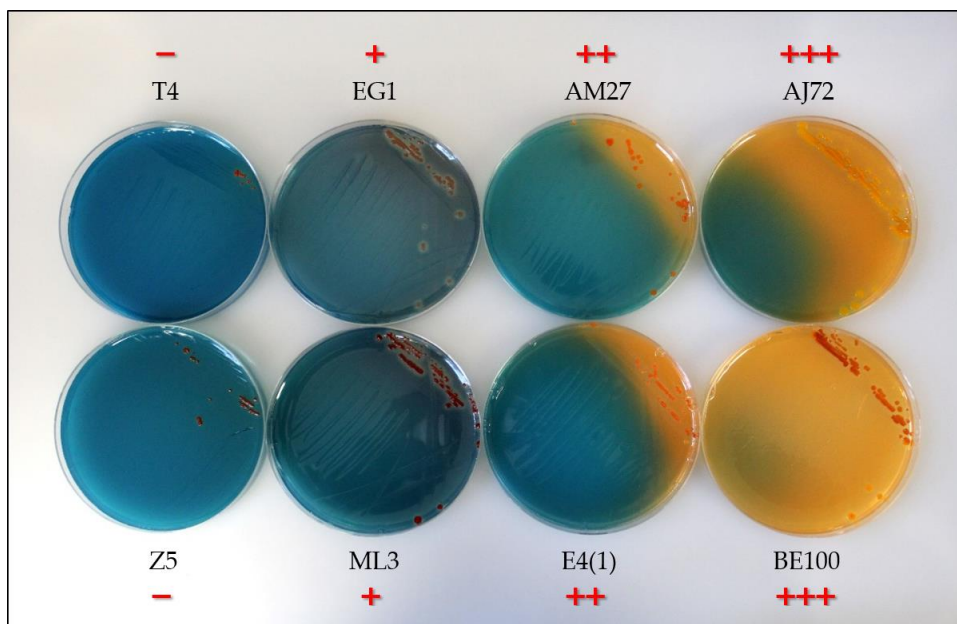


Fig. 5.3. Iron chelating CAS assay after 5 days' growth. Zones of activity are separated into 4 groups based on range of colour change, as indicated in red by each strain name.

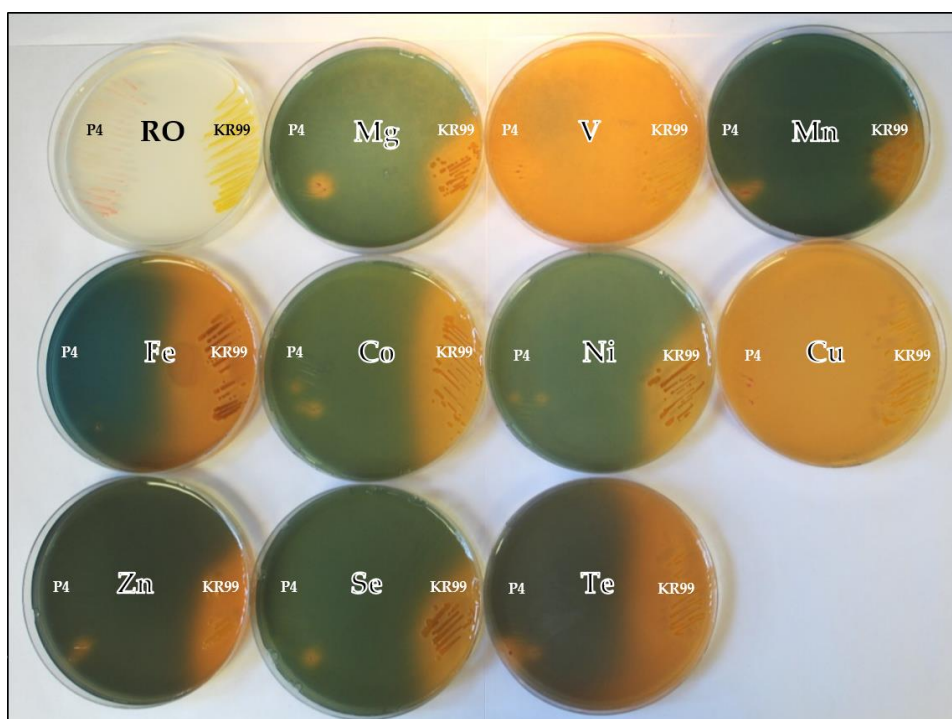


Fig. 5.4. Variant metal(loid) CAS assay after 5 days' growth for strains P4 and KR99^T. Growth on RO without CAS was compared to those CAS plates supplemented with Mg, V, Mn, Fe, Co, Ni, Cu, Zn, Se, or Te.

Tables 5.3 and 5.4 using the same zone distinctions as described. While some strains only produced siderophores that reacted with Fe^{2+} , others had secondary metabolites capable of chelating additional metal(loid) cations. Most strains capable of chelating metals other than Fe could also chelate iron, with the exception of two strains P4 and SS56 (Fig. 5.4), which were found to produce metallophore capable of acting predominantly on other tested metal(loid)s, rather than Fe.

5.5.3. *Phylogenetic diversity of siderophore producing AAP*

Isolates were chosen to represent AAP from a variety of environments as well as embody a host of phylogenetically diverse species. In this way, the 101 representatives listed in Tables 5.3 and 5.4 were cultivated, and 16S rRNA gene sequences acquired either from repositories, or decoded in this work. Phylogenetic relation to sequences of known type strains was determined by BLAST search (Tables 5.1 and 5.2). In addition, these sequences were used to create a phylogenetic tree (Fig. 5.2), that also included some previously described type species not tested for siderophore production, but were included as key anchors of phylogenetic groups, as described AAP type species. The evolutionary analysis performed on Mega X using Maximum Likelihood method involved 132 nucleotide sequences and had a total of 1717 positions in the final data set. The chosen AAP diversely spread throughout *Erythrobacteraceae* and *Sphingomonadaceae* relating to known AAP type species. While some aligned to reported AAP in *Acetobacteraceae*, and *Rhodobacteraceae*, many others aligned to organisms previously undescribed as AAP within these clades, as well as to some species within *Hyphomonadaceae* and *Methylobacteraceae*.

5.5.4. *C. halotolerans pigment purification and identification*

When purified via 16.5% tris-tricine gel electrophoresis, the brown pigment migrated further than the loading buffer's running dye, CBB, after 1 h (Fig. 5.5A). This gel-shift revealed the pigment under study to be smaller than CBB, which has a known molecular weight of 856.03 g/mol. Since well 1 contained a standard ladder, the measurement of migration distance for the siderophores' brown band, and of each protein in the ladder allowed for the rough estimation of pigment size to be near ~341 Da (Fig. 5.7). Gel staining and destaining revealed that the siderophore sample collected after resin concentration and diluted in methanol at 200, 20, 2, and 0.2 $\mu\text{g/mL}$ loaded into wells 2 through 5, respectively, had some contaminating small proteins as expected (Fig. 5.5B). In addition, samples that received the subsequent removal of proteins larger than 3 kDa via spin-column were also run on the same gel. Wells 6 through 8 contained brown pigment, which passed through the <3 kDa spin column and then diluted in 60% methanol to 100, 10, and 1 $\mu\text{g/mL}$, respectively. This step purified the brown pigment of any contaminating proteins (Fig. 5.5A and B). Often,

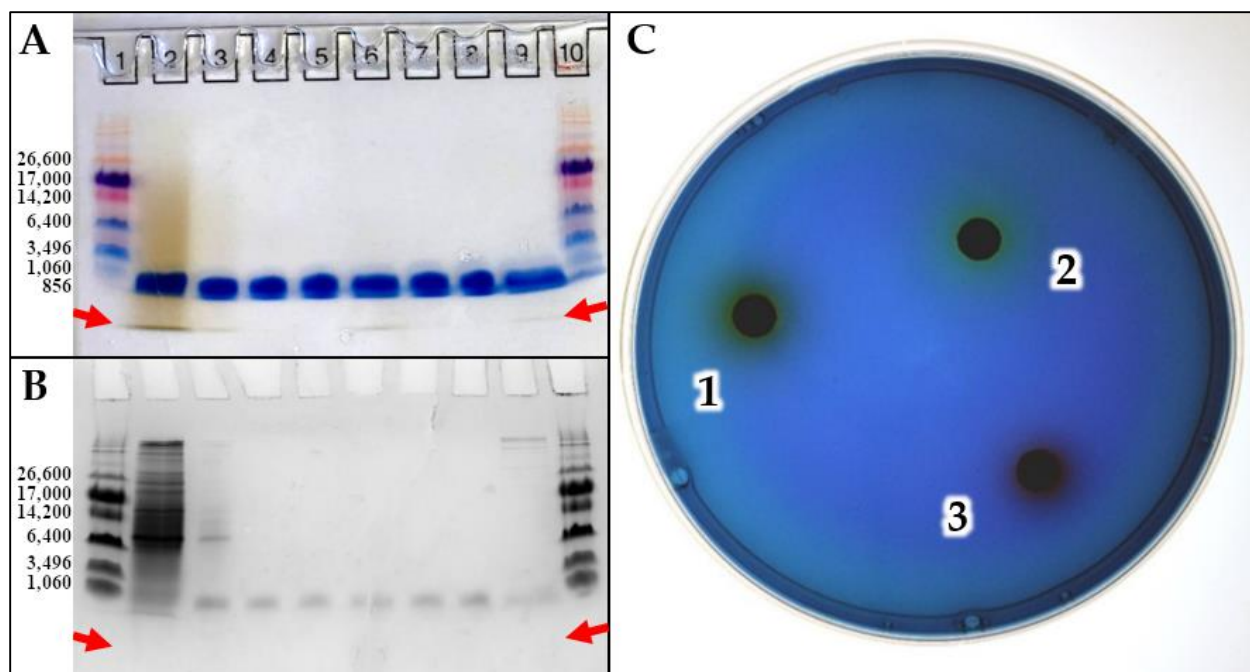


Fig. 5.5. Gel purification of *C. halotolerans* brown pigment, and confirmation of siderophore activity. (A) Unstained and (B) stained tris-tricine gel electrophoresis performed on: siderophore from resin concentration, wells 2–5; siderophore sample smaller than 3 kDa, wells 6–8; proteins larger than 3 kDa and remaining in solution well 9; standard ladder, wells 1 and 10. (C) Siderophore activity observed for: (1) Crude resin extract positive reaction visible as yellowing area; (2) <3 kDa dark brown fraction produced positive yellowing reaction; (3) Proteins >3 kDa negative results observed as darkening of blue due to alkaline pH, without siderophore activity present.

small molecules below 1 kDa are lost from the gel during destaining step (Schägger and von Jagow, 1987), which likely caused the small brown pigment to escape similarly to CBB, (Fig. 5.5B). Regardless, testing the crude dried pigment (Disk 1), as well as fractions <3 kDa and >3 kDa (Disks 2 and 3), confirmed the smaller fraction containing brown pigment acted as a siderophore, while the larger proteins did not (Fig. 5.5C). Since the <3 kDa fraction had no contaminants (Fig. 5.5B), but contained the brown pigment prior to destaining (Fig. 5.5A), the small approximately ~341 Da molecule produced by *C. halotolerans* acted as a siderophore.

5.6. Discussion

5.6.1. Siderophore production revealed by CAS-assay

While effectively employed for the identification of numerous siderophore producing bacteria, the CAS assay has a notable limitation; microbial growth may be hindered due to a few factors (Schwyn and Neilands, 1987). Since the metal cation of interest weakly bound to the dye, it was less freely diffusible into the bacteria, resulting in lower availability. In addition, the medium needed to be at pH 6.8 for the indicative colour change to occur successfully. Finally, HDTMA has been known for its slight bacterial toxicity. If an organism had weak Fe transport, required a basic or acidic optimal pH, or was sensitive to HDTMA, it would have had reduced growth. In our experiments, we found that while a range of media compositions could be used, all AAP did have reduced growth on CAS plates, when compared to control. However, since growth did indeed occur, siderophore production could therefore be analyzed.

Other, more high-throughput, alternative methods were considered, but the chosen agarized CAS-assay was most ideal for determining siderophore and metallophore production. Recently, a bulk screening assay for siderophore detection was proposed (Arora and Verma, 2017). However, both the “traditional” and “modified microplate” qualitative techniques described could not be used for our application, since there was an assumption that siderophores were always constitutively expressed, whereas cultures were grown in complex media without manipulating the concentration of any metal of interest. While this may be the case for some strains, the expression/production of most siderophores or other secondary metabolites usually requires induction from an external factor, which can be either the presence or absence of a stimuli (Pedler *et al.*, 2000). For biologically significant metals, including Fe, Zn, and Cu, the related cation-specific metallophores are expected to be produced only under limiting conditions. In opposite, metallophores that act on V, Te, Se, or other more toxic metal(loid)s are presumably only synthesized when such toxins are present at higher concentrations. Therefore, growth on agarized plates that contained each metal of interest pre-bound to CAS-reagent as a stimulant for metallophore production was our chosen method. Future works

are required to test varying concentrations of metal cations to determine which yields more/less production of specific metallophores.

Regarding the Fe-chelation, a range of phenotypes was observed, when detecting siderophore activity after the 5-day incubation (Fig. 5.3). In particular, 4 phenotypes were distinguished among all isolates based on size of clearing/colour change zone. Negative results (–) had to have bacterial growth, but without a change in medium opacity. The smallest zone of clearing (+), seen previously after prolonged growth (Ames-Gottfred *et al.*, 1989), was likely not due to siderophore production (Amaro *et al.*, 1990). Rather, this small aura could be due to high rates of metal uptake from the surrounding medium. Prolonged bacterial metabolism allowed for increased simple metal diffusion into cells, which reduced its amount in the near-by medium, rendering the dye in that narrow area void of metal, turning yellow. Hence, there is a very small <1 mm zone. In comparison, moderate or highly diffusible siderophore release was quite evident, and was segregated into two phenotypic groups. A zone <10 mm compared to a zone >10 mm, where each represented less or more diffusible secondary metabolite, respectively, and where lower or higher concentrations were produced and captured additional metal cations. In this test, all 4 zonal varieties for Fe-chelation were discovered (Tables 5.3 and 5.4, and Fig. 5.2).

Modifying the CAS assay to monitor chelation of elements other than Fe^{2+} included Mg^{2+} , V^{3+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Se^{4+} and Te^{2+} (Fig. 5.6A). The method could be successfully adapted for all selected metal(loid)s, where only Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} had been proposed previously (Patel *et al.*, 2018). In addition, the assay could be used with variable nutrient and organic carbon concentrations that did not inhibit the activity. Furthermore, we discovered that a wide range of AAP produced metallophores, which bound a variety of metal(loid)s in addition to Fe (Tables 5.3 and 5.4). Some AAP had siderophores that specifically bound Fe only, including strains E1, E4(1), RB3, NM416, AM27, CK155, BC100, Z24, Z39, and J05. Most AAP with highly diffusible siderophores, and >10 mm zones on Fe-CAS plates, had activity towards a large variety of metal(loid)s. Few strains, including SS56 and P4 preferentially bound metal(loid)s other than Fe. Since all 9 additional cations Mg^{2+} , V^{3+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Se^{4+} and Te^{2+} could be bound by metallophores, future work may consider a broader range of cations and the extent of metals that can be exogenously chelated. To determine if specific metal(loid)s were more readily chelated than others, all positive CAS assay results were tallied for each, Fig. 5.6B. Here, multiple +++ represented strong production or significant interaction, and could be compared to weaker reactions such as +, or ++. In this way, Fe was

the highest cumulative acquired cation, with the activity ranked as Fe > Zn > V > Te > Cu > Mn > Mg > Se > Ni > Co. It would appear slight variation in cation size, from Fe (55.85) to Co (58.93), had a strong impact on activity, where Fe was most frequently captured, and Co the rarest. Indeed, since strongest reactions existed for Fe and Zn, with less reactivity found for metals in between the sizes of these two within the periodic table, specific mechanisms likely existed to capture either metal, inferring cation specificity for each metallophore. Further analysis will be required to see if the broad range metal(loid) acquisition is due to the production of a single siderophore that reacts with a variety of metals, or the bacteria produced specific metallophores for each. AAP metallophore production could be explained by requirement of trace elements and the need of toxicity prevention. Both Fe and Zn are biologically necessary and commonly limited or unavailable in dissolved forms, therefore acquisition via specific siderophores would be an asset. The 3rd and 4th highest captured metal(loid)s were V and Te, which have known toxic properties, and could have been sequestered as a result of a protection mechanism alone. Bound to a metallophore, these cations would be too large to freely diffuse through outer membranes and be restricted from entering the cell, to prevent any toxic influence. The remaining 6 had reduced activity, likely because they are less toxic and easier available in microbial environment, and therefore less metallophores could be expected.

5.6.2. *Environmental distribution of siderophore producers*

In relation to the origin of isolation a few patterns were observed. Collections of AAP strains that originated from hot springs, freshwater lakes, and biological soil crusts all had a high proportion of siderophore producers. In opposition, the isolates from marine, meromictic lake, and saline spring habitats produced less, or no siderophores. Rather, they seem to rely on sufficient metal uptake directly from the local microenvironment by diffusion. The main differentiating factor in this case is the requirement of NaCl. It appears that AAP capable of halotolerance or halophilic growth do not produce siderophores of equal activity or quantity as bacteria that do not depend on NaCl for growth. This correlation may be due to each strains' reaction to osmotic pressure. Cells that are adapted to tolerate higher levels of NaCl will likely have additional cation pumps to survive naturally and tolerate the high levels of solutes in a saline environment. As seen elsewhere, cation pumps can be non-specific, where many are capable of removing several toxic cations from the cytoplasm of microorganisms (Rensing *et al.*, 1999). In comparison, freshwater AAP are comparably less osmophilic, and would therefore have less need of copious cation pumps in membranes. Therefore, they are more likely to evolve defensive mechanisms that modify the local environment to suit their needs, including the production of external small molecules that would render cations less diffusible.

5.6.3. Phylogenetic diversity of Fe-chelating AAP

Comparing phylogenetic diversity and the production of siderophores on CAS plates by AAP delivered a few notable trends (Fig. 5.2). Broadly, no studied representatives of the order *Rhodobacterales* or *Hyphomonadaceae* had siderophores, as no or <1 mm zone of colour change was present. The *Acetobacteraceae* that were in closest relation to known type species of AAP, strains RB-3^T and CK155 had siderophores that only chelated Fe as discussed above. In comparison, strains P40 and Jo1 were more genetically distant from known type species of AAP, and also produced significant zones of clearing, ≥10 mm for all metal(loid)s tested, signifying their own group. The predominantly strong expression of siderophores by AAP among the *Methylobacteraceae* warrant further study as most type species in this clade have not been previously recognized as phototrophs. Of note, previous research had found that isolates relating to *Methylobacterium mesophilicum* and *M. extorquens* produced siderophores, but the activity had not been linked to aerobic anoxygenic phototrophy (Idris *et al.*, 2004). One clade of AAP which related by 98.7–99.1% 16S rRNA sequence similarity to *Bosea lupini*, including strains P13, SS335, and SS63 were all capable of strong production of metallophores, >10 mm zones for multiple metal(loid)s. In comparison, strains P4 and SS56, which have 99.6% relation to *M. phyllosphaerae* and 99.6% to *M. branchiatum*, respectively, both had stronger reactions against metal(loid)s that were not Fe (Fig. 5.4). This activity may be explained through the findings of related works, where methanotrophs produced methanobactin, a chalkophore, which is a Cu specific metallophore (Kim *et al.*, 2004). The isolates we have tested may indeed possess similar mechanisms as they sequester Cu strongly, as well as Mg, V, Mn, Zn, and Te more favorably than Fe.

Sphingomonadaceae could be separated into 3 groups, as those related to *Citromicrobium* did not produce siderophores, *Sphingomonas* relatives produced some siderophores that were predominantly Fe specific, while relatives of *Blastomonas* could produce siderophores that acted on all metal(loid)s tested. A few *Sphingomonas* relatives had been previously found to produce siderophores against Fe (Sun *et al.*, 2010), but not other metal(loid)s. The *Blastomonas*/*Erythromonas* grouping was of particular interest as most representatives revealed strong metallophore production against all 10 cations tested. Our results corresponded well with previous analysis of *E. ursincola*, strain KR99^T, which had very high tolerance to V, Te, and Se oxides, internally reducing them to elemental states (Maltman *et al.*, 2017). Since KR99^T can both acquire Se, Te, and V via metallophore activity (Fig. 5.4), and internally reduce metal(loid) oxides, it appears to require them in reduced form for some reason. Future study of such associations will determine if *E. ursincola* sequesters these cations as a protective measure, or uses them for some metabolic purpose. In

comparison, the *Erythrobacteraceae* were not as concisely divided as other families, where those closest to *Erythromicrobium* had siderophores, but most *Porphyrobacter* had very small <1 mm or negligible zones. An exception was strain BE100, which branched distantly from its nearest relative *P. colymbi* (Fig. 5.2), and showed a significant presence of metallophores, ~10 mm zones for all metal(loid)s except Te. Finally, strain EG19^T that hailed from γ - rather than α -Proteobacteria, had moderate siderophore production for both Fe and Zn, <10 mm. While many siderophores have been discovered as products of bacteria within the γ - proteobacterial clade (Table 5.5), none have been documented as highly pigmented.

5.6.4. Analysis of the brown-coloured siderophore

Gel purification of brown pigment produced by *C. halotolerans* (Fig. 5.5), revealed that while CBB bound to proteins remained in the gel, both unbound CBB and brown pigment were released during destaining process. Comparing lanes 2–5, the sample prepared by resin concentration clearly contained brown pigment, but also accumulated proteins smaller than 26 kDa. The use of the 3 kDa cut off spin column did indeed remove these contaminants, as shown in lanes 6–8. Since the small brown pigment passed through the spin column, was purified on gel electrophoresis, and maintained activity on CAS plates, these tests confirmed that it acted as a siderophore. Furthermore, these procedures established the brown compound to clearly be under 800 Da, and approximated to be around ~341 Da when correlated to the ladder during TRIS-tricine gel electrophoresis (Fig. 5.7). Both the estimated small size and the brown appearance of the siderophore synthesized by *C. halotolerans* were useful for its tentative identification. The most comparable small molecule described in literature was rhodotorulic acid (Table 5.5), a 344.4 Da siderophore that was pigmented red when bound to Fe (Atkin and Neilands, 1968). However, this acid has only been naturally found in yeasts including *Rhodotorula pilimanae*, with no known bacterial producers (Andersen *et al.*, 2003; Holinsworth and Martin, 2009). With that in mind, hydroxamic acids are produced by both bacteria and fungi (Carson *et al.*, 2000), and therefore similar secondary metabolites can be expected in other species. In addition, the colour disparity, red compared to orange-brown, may indicate an altered structure among siderophores produced by *R. pilimanae* and *C. halotolerans*, respectively.

Since *C. halotolerans* hails from the γ -Proteobacteria, comparisons must be drawn between its siderophore and those produced by other species in the γ -subclass. The most similar in size was acinetobactin, a 346.4 Da molecule from *Acinetobacter baumannii* expressed using the operon containing *basABCDEFGHJJ*, *bauABCDEF* and *barAB* genes (Mihara *et al.*, 2004). *C. halotolerans* genome, published

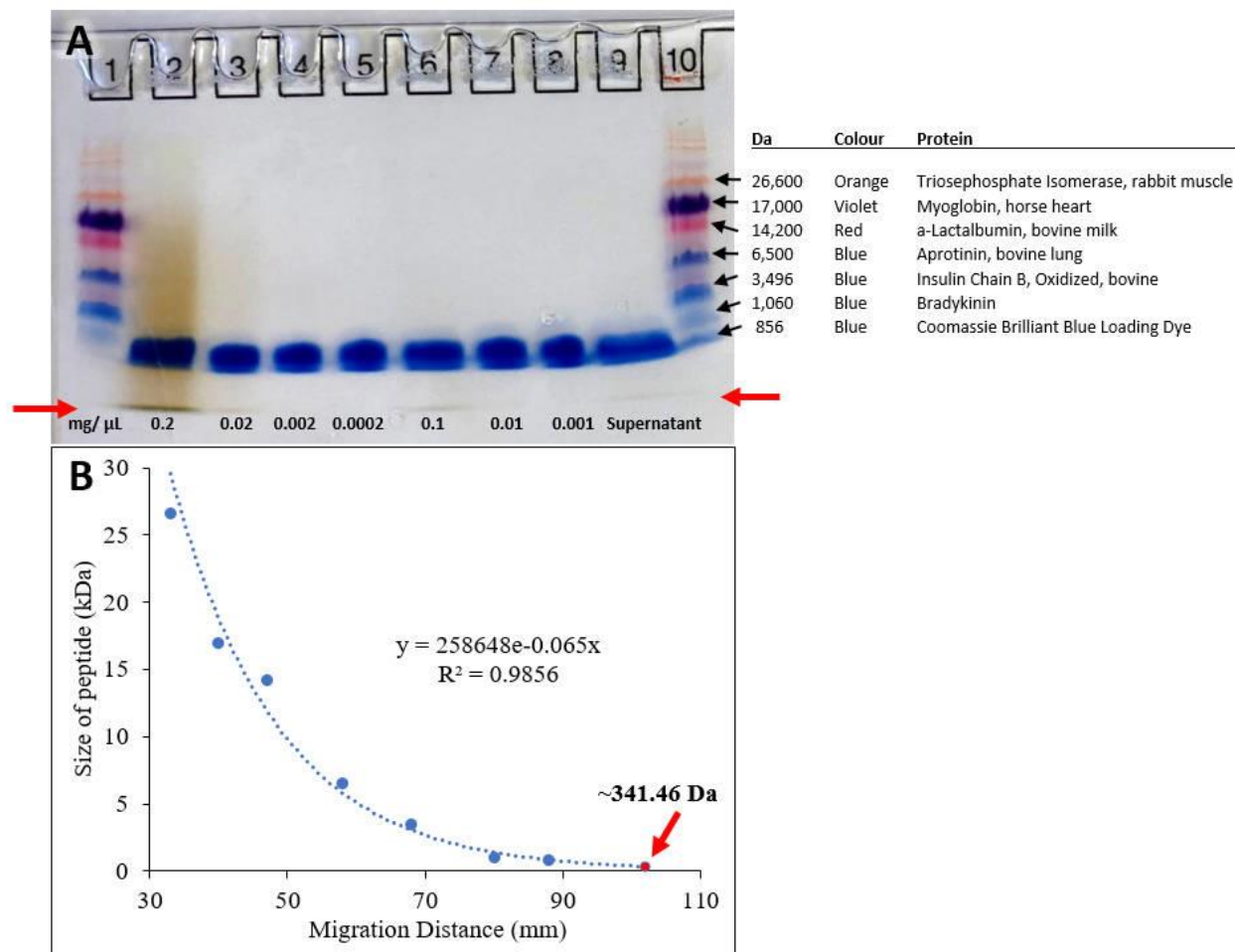


Fig. 5.7. Approximation of siderophore size. (A) Sample and standard ladder run on tris-tricine gel had migration distances measured, noting size of each known protein. (B) Standard curve of migration distance compared to known sizes used to calculate brown pigment weight of ~ 341.46 Da.

Table 5.5. Siderophores examples listed from largest to smallest.

Siderophore	Size (Da)	Species that produces	Phylum
Pyoverdine	1365.4	<i>Pseudomonadaceae</i>	γ -Proteobacteria
Alterobactin B	945.9	<i>Pseudoalteromonas luteoviolacea</i>	γ -Proteobacteria
Alterobactin A	927.9	<i>Pseudoalteromonas luteoviolacea</i>	γ -Proteobacteria
Ferrichrome	740.5	<i>Aspergillus, Ustilago, and Penicillium</i>	(Fungi)
Enterobactin	669.6	<i>E. coli and Salmonella typhimurium</i>	γ -Proteobacteria
Acinetobactin	346.4	<i>Acinetobacter baumannii</i>	γ -Proteobacteria
Rhodotorulic acid	344.4	<i>Rhodotorula pilimanae</i>	(Yeast)

within the One Thousand Microbial Genomes Phase 4 (KMG IV) project by the DOE Joint Genome Institute, submitted online in 2019 with accession number PRJNA520330 (Goeker, 2016), contained neither similar genes nor was the operon present, while using very low homology search. Due to the divergence between *C. halotolerans*' pigmented siderophore and *A. baumannii*'s lack of colour, and the absence of similar genes, we assume that acinetobactin was not the siderophore of *C. halotolerans*. Further structural analysis will be necessary to confirm the structural identity of this novel compound.

5.7. Conclusions

We have discovered that many AAP produce siderophores or metallophores as diffusible secondary metabolites. Production could be related to acquisition of metal(loid)s including magnesium, vanadium, manganese, iron, cobalt, nickel, copper, zinc, selenium and tellurium, or to provide tolerance to toxic metal(loid)s in environments with elevated concentrations. A correlation existed between site of isolation and production of siderophores, whereas tested freshwater AAP produced siderophores, and AAP that required NaCl predominantly did not. Furthermore, there could be connection between phylogeny of isolates and their ability to form siderophores, but as with many phenotypes to genotype comparisons, it did not appear as a strictly followed rule. With such considerations, siderophore production cannot be recommended as a taxonomic marker for AAP identification, as variable production types occurred. However, a potential application exists to use this phenotypic feature during taxonomic differentiation between species. Future work will hopefully identify the siderophores, and potential metallophores, produced by each AAP, and determine the total list of metal cations that can be targeted.

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6. Chapter 6.

Psychrotrophic violacein producing bacteria isolated from Lake Winnipeg, Canada

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6.0. Preamble

As we were studying pigmented bacteria from Lake Winnipeg, we found isolates of various colour worth investigating. Of those, the rarest and most abnormal colouration found, was violet. Such a hue is extremely rare in aerobic microorganisms. We investigated these bacteria in hope of identifying the nature of such pigmentation, finding it to not be from bacteriochlorophyll mediated anoxygenic photosynthesis, but rather, from the unusual membrane bound pigments violacein and deoxyviolacein.

6.1. Contributions statement

Conceptualization, S.B.K. and V.Y.; methodology, S.B.K., A.O.P., J.L.S. and V.Y.; software, S.B.K., A.O.P., J.L.S. and V.Y.; validation, S.B.K., A.O.P., J.L.S. and V.Y.; formal analysis, S.B.K., A.O.P., J.L.S. and V.Y.; investigation, S.B.K., A.O.P., J.P., J.L.S. and V.Y.; resources, J.L.S. and V.Y.; data curation, S.B.K.; writing—original draft preparation, S.B.K.; writing—review and editing, S.B.K., A.O.P., J.L.S. and V.Y.; visualization, S.B.K., A.O.P., J.L.S. and V.Y.; supervision, J.L.S. and V.Y.; project administration, V.Y.; funding acquisition, J.L.S. and V.Y.

S.B.K. isolated the violet strains, and discussions between S.B.K and V.Y. led to the suggestion of violacein production and its later structural analysis. All physiology, phylogeny, formal descriptions and analysis were done by S.B.K. Production of violet pigment and initial preliminary purifications were performed by S.B.K. and J.P. and finalized by S.B.K.. Collaboration with A.O.P. and J.L.S., who further purified pigments using specialized equipment and decoded structures of small molecules of violacein and deoxyviolacein (Methodology in Section 6.4.6). The first manuscript draft was written by S.B.K., who also designed, created and curated all figures.

6.2. Abstract

Violet and purple bacterial pigmentations are uncommon when considering strict aerobes. Here we report discovery of two novel isolates, P102 and P117 from the freshwater Lake Winnipeg, each with violet to deep purple colony colouration. The relationship between pigment production and growth was investigated under different conditions, including a variety of carbon sources, pH, and temperatures. The violet compounds were purified using polar organic solvents, and then structurally characterized via mass spectrometry and nuclear magnetic resonance to be violacein and deoxyviolacein. Strain P117 produced higher concentrations of the darker pigment deoxyviolacein, which resulted in a deeper hue of violet than in cultures of P102. Strain P102, synthesized violacein maximally in liquid rich organic medium at pH 8 and 20°C, and had 99.3% sequence similarity to the 16S rDNA from *Janthinobacterium lividum*. Strain P117, related to *Massilia violaceinigra* sharing 99.2% 16S rDNA sequence similarity, also produced violacein at similar optimal conditions, but developed higher concentrations of pigment at 15°C. Culture-based methods found that violacein producers composed 0.001% or 0.023% CFU of environmental heterotrophic populations using BG-11 and potato-based media, respectively. Culture-independent high-throughput ribosomal 16S V₄ sequencing of environmental DNA was used to detect Gram-negative species known for their production of violacein. Of all bacterial and archaeal sequences present during the fall in littoral waters and sediment of Lake Winnipeg, 5.5% and 6.3%, respectively, belonged to species that have similar pigment, demonstrating the scarcity of violacein producers in this aquatic freshwater system.

6.3. Introduction

Cellular bacterial pigmentation is routinely used for taxonomical differentiation and identification of species. However, the environmental factors promoting internal colouration and associated biological functions have only intermittently been determined (Venil *et al.*, 2013). Phototrophs have been generally regarded for their known colour determinants, where red, pink, yellow, and orange appearances were typically due to the synthesis of carotenoids (Yurkov and Csotonyi, 2009). Green has been associated with chlorophyll a production in oxygenic cyanobacteria or a simultaneous presence of carotenoids and bacteriochlorophyll in some anoxygenic phototrophs (Marosvölgyi and Gorkom, 2010). While purple cells are common primarily among anaerobically growing phototrophic purple sulphur and non-sulphur bacteria (Henry and Cogdell, 2013), blue has been observed within the single Gram-negative heterotrophic species *Pseudomonas aeruginosa*, connected to a secondary metabolite pyocyanin (Baron and Rowe, 1981). In contrast, violet and purple pigments have not often developed under aerobic conditions, especially within heterotrophic bacteria (Soliev *et al.*, 2011). In rare cases, cellular violet appearance has been attributed to the production of violacein in Gram-negative β -*Proteobacteria*, among the family of *Oxalobacteraceae*, including the genera *Janthinobacterium*, *Collimonas*, and *Duganella*, as well as *Chromobacterium* spp. in the family of *Neisseriaceae*. The γ -*Proteobacteria* *microbulbifer* and *Pseudoalteromonas* also produced this natural colourant (Ahmad *et al.*, 2011). Meanwhile, fungi grown in aerobic environments create a broader range of purplish secondary metabolites, including rubropunctamine, monascorubramine (Prabhu *et al.*, 2017), xanthomegnin, viomellein, and viopurpurin (Stack *et al.*, 1997). Some other eukaryotes synthesize anthocyanins (Kim *et al.*, 2012), but none of these have been found in bacteria to date (Hidalgo *et al.*, 2012; Khoo *et al.*, 2017).

All currently known violacein-containing species are heterotrophic, and have been predominantly discovered within aquatic ecosystems. Furthermore, many representatives have been isolated from cold environments, such as arctic ice, freshwater and marine habitats, and are therefore slow growing even under optimal conditions (Choi *et al.*, 2015a,b). While this pigment is found in a variety of microbes, its biological function remains to be determined. In laboratory conditions, violacein has had effective anti-eukaryotic and antibacterial properties, inhibiting growth of fungi (Brucker *et al.*, 2008), nematodes (Ballestriero *et al.*, 2014), and some Gram-positive bacteria like *Staphylococcus aureus* (Im *et al.*, 2017). However, due to the exclusively intracellular membrane localization of violacein, according to the current hypothesis (Choi *et al.*, 2020), its production in nature is likely related to a defence mechanism against grazing nematodes, or faster

growing organisms within proximity by shedding vesicles laden with this small molecule that will disrupt neighboring membranes. Violacein also has potential applications in biotechnology, both as a natural industrial colourant due to its deep hue and stability (Kanelli *et al.*, 2018), and for its anticancer effects (Choi *et al.*, 2015a).

Investigation of pigmented bacteria in Lake Winnipeg, which is located north of the 49th parallel in Manitoba, Canada, and is the eleventh largest fresh water lake in the world, led our group to find aerobic violet strains. Due to its location, Lake Winnipeg and its watershed have a cold continental climate, subjected to drastic temperature changes (Kottek *et al.*, 2006). Ice covers parts of the surface for over five months of the year, and mean water temperatures fluctuate between 21.5°C in the summer, and near 0°C under the ice in the winter (MWSD, 2011). It is eutrophic due to nutrient run off from its large drainage basin, ~1,000,000 km² dominated by agricultural activity. The enumeration of bacteria with an internal violet hue, the characterization of pigments, and conditions of optimal production, were the focus of our research.

6.4. Materials and Methods

6.4.1. Sample collection, media, and bacterial enrichments

To assess aerobic violet pigment producers, 13 locations across Lake Winnipeg were monitored between 2017 and 2019 (Fig. 6.1). Samples were collected via road access to South Basin shoreline littoral zones, Sites 1–4, or via helicopter to limnetic zones (Site W6) during winter months, when the lake was frozen over. Temperatures in water and ambient air, as well as pH were recorded at the time of collection. Strain P102 was isolated in early May 2017 from sediment under 0.5 m depth littoral shore water at S2, Patricia Beach, 50°25'25" N, 96°36'60" W. During October 2017, P117 was cultivated from littoral water of S4, near 50°37'11" N, 96°18'55" W. In both cases, liquid samples were transported back to laboratory in sterile containers on ice within 24 h for enrichment and enumeration. Samples were serially diluted, and 100 µL from each dilution was spread on 2% agar plates. P102 was initially cultivated on a modified BG-11 medium (Bilyj *et al.*, 2014), that contained the original components (Waterbury, 1992), with the addition of trace elements and vitamins to help cultivate auxotrophic organisms (Yurkov *et al.*, 1999). P117 was purified from agarized potato broth (PA) medium (Yurkov, 2006). Violet-purple colour was observed in cells of both isolates after growth for 7 days at 28°C in the dark. Further purification steps were conducted with rich organic (RO) medium free of NaCl (Yurkov *et al.*, 1999), as growth rates in RO were higher than on BG-11 or PA, and its composition was not inhibitory for pigment synthesis.



Fig. 6.1. Lake Winnipeg Sampling Sites. S1-S4 were accessed by road during thawed colder months to retrieve water samples. Site W6 was accessed via helicopter during frozen months to retrieve water from under ice. Red dots indicate locations sampled, but no violacein producers were detected.

6.4.2. Phylogeny of violet-coloured isolates

Upon isolation and purification of bacterial strains, DNA was chloroform extracted (Rainey *et al.*, 1996). Ribosomal 16S rDNA reads were then procured with Sanger sequencing method using the universal primer set 27F and 1492R, 5' -AGAGTTTGATCCTGGCTCAG-3', and 5' -GGTTACCTTGTACGACTT-3', respectively. Sequences of strains P102, P117, P016, P110, P063, P034, P035, and P041 have been deposited in Genbank with accession numbers MN987201-MN987208, respectively. Phylogenetic relationships of isolates to known bacterial species were then determined with web-based Nucleotide Basic Local Alignment Search Tool, BLAST (Madden, 2002). Phylogeny was established using MEGA 10 software (Kumar *et al.*, 2018). Molecular phylogenetic analysis was performed following the maximum likelihood method based on the Jukes-Cantor model with 10 K bootstrap iterations (Jukes and Cantor, 1969). Initial trees for the heuristic search were obtained automatically by applying neighbor-joining and 'BioNJ' algorithms to a matrix of pairwise distances estimated with the Maximum Composite Likelihood (MCL) approach. Topology was then selected for superior log likelihood value.

6.4.3. Environmental analysis of violacein producers based on 16S rDNA sequences

As in co-published work, environmental DNA was extracted from each site using a QIAGEN DNeasy PowerWater Kit method with minor modifications (Chapter 2,3). Ribosomal 16S V4 amplicon gene sequencing was completed by MiSeq Illumina with the primer set 515F paired with 806R; 5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTCTAAT-3', respectively. Community structural analysis was carried out via Qiime2 computational pipeline, using Python based (McKinney, 2010) framework (Bolyen *et al.*, 2019), and BIOM format (McDonald *et al.*, 2012). Methods included importing raw data, joining paired end reads, deblurring (Amir *et al.*, 2017), and denoising (Bokulich *et al.*, 2013) prior to operational taxonomic unit (OTU) alignment (Price *et al.*, 2010; Rognes, *et al.*, 2016). Three clustering methods were compared: *de novo*, open-reference and closed-reference using the Silva132 database and 99% identity (Quast *et al.*, 2013). Sequences of borderline-chimeras were considered in analysis as they provided a larger diversity in samples. Phenetic identities were aligned as feature tables (Weiss *et al.*, 2017) at all levels of the phylogenetic tree (Stackebrandt and Goodfellow, 1991; Katoh and Standley, 2013). Diversity was statistically quantified via α - and β - diversity methods (Faith *et al.*, 1987; Vázquez-Baeza *et al.*, 2017). Raw sequencing data has been submitted to the National Center for Biotechnology Information Sequence Read Archive under Bioproject PRJNA603112.

Pseudoalteromonas leteoviolacea (Gauthier *et al.*, 1995), *Microbulbifer* sp. (Matz *et al.*, 2008), *Chromobacterium violaceum* and *C. pseudoviolaceum* (Kämpfer *et al.*, 2009), *Collimonas* sp. (Hakvåg *et al.*, 2009), *Duganella violaceinigra* (Choi *et al.*, 2015b), *Massilia violaceinigra* (Wang *et al.*, 2018), and *Janthinobacterium lividum* (Pantanella *et al.*, 2006) all produced violacein. Sequencing information from genes of these recognized organisms was used to determine the prevalence of violacein producers within the natural bacterial community under investigation.

6.4.4. Physiology of new isolates

Carbon source utilization was studied as described in Chapter 3 and 4. For each test, 2% carbon source was provided, followed by adding a 5% inoculum, prior to a 7-day incubation at either 20 or 25°C. In addition to the impact of carbon sources on growth and pigmentation, the effect of temperature was determined by cultivating bacteria for 7 days in liquid RO using incubators that provided cooling as low as 4°C or heating to 50°C. To examine growth below 15°C, incubations continued for an additional 7 days to demonstrate psychrotolerant capabilities of strains. Following the determination of optimal temperature for cultivation, strains were tested for their capability to grow and produce violet pigment over a range of pH. RO medium was adjusted with concentrated HCl or NaOH to a point between the range pH 5 and 12, prior to inoculation of either strain, and then incubation for 7 days at 20°C. All tests were performed in triplicate, with 6-in. test tubes filled with 8 mL of medium and shaken at 100 rpm in the dark. Measurements were taken at time zero and at the end of each incubation to infer growth and production of pigments for comparison between all conditions. Bacterial concentrations were estimated via optical density (OD), and pigments were detected spectrophotometrically on a HITACHI U-2010, both in a 10 mm path length cuvette. Cellular OD was measured at 660 nm, red shifted from the standard 600 nm, to avoid the influence from the violet hue absorption, which tailed off near 600 nm. For pigment quantification, 1 mL of culture from each time point was centrifuged at 20,000g for 30 min. The supernatant was discarded, and pigments were extracted from the pellet in 0.5 mL acetone for 1 h in the dark. Cell debris was spun down at 20,000 g for 10 min and removed, followed by absorption spectra read between 350 and 1100 nm, revealing a peak at 560 nm. Absorbance readings at 560 nm were therefore used for pigment quantifications in extracts from both isolates.

The optimal growth rate of isolates was analyzed at 15, 20, or 28°C within $\pm 0.1^\circ\text{C}$. To keep cultivation environments consistent, 5% inoculum was added to 1 L of medium, in triplicate, which was subsequently

aliquoted into 8 mL test tubes at time zero. These were then incubated at selected temperatures, and individual tubes could be removed for measurements at each time point, in triplicate. In this way, temperature and volume of the remaining experimental cultures would stay the same to complete the growth curve.

6.4.5. Extraction and purification of pigments

Pigments were extracted in bulk as previously described (Yurkov *et al.*, 1993). In brief, strains were grown 5–7 days, until violet colour developed. Cell pellets were obtained by either scraping biomass off agar plates, or spinning down liquid cultures at 17,600 g for 30 min, followed by resuspension in 50 mL spent media, prior to another centrifugation at 30,000 g for 30 min. Supernatant was decanted prior to addition of organic solvents, all purchased from Sigma-Aldrich Canada, to test the crude extraction of pigments. These included polar solvents acetone, methanol, or ethanol, as well as mixtures of acetone and methanol at ratios of 7:2 or 1:1. Non-polar solvents tested were chloroform or diethyl ether. Coloured products from pellets leached into polar solvents during overnight extractions at 4°C in the dark. Cellular debris was spun down at 30,000 g for 30 min, and pigment-containing supernatant was collected for spectrophotometric analysis on a HITACHI U-2010 and for further processing. The stability of extract colour under acidic and basic conditions was analyzed (Ahmad *et al.*, 2011). Crude extract of P117 in acetone started at pH ~ 7.5, which was gradually decreased to near 1.0 with the addition of concentrated HCl, or increased to ~13 with granulated NaOH. The change in absorbance spectrum and colouration were monitored at each pH value.

For thin layer chromatography (TLC), pigments were extracted from cells in acetone. The resulting extract was collected in brown light-protecting glass bottles and concentrated by enhanced evaporation (Mahon, 1997). Repetition allowed sufficient pigment concentrate to be obtained, and it could be stored in the dark at 20°C if necessary. Silica gel 250 mm thick on polyester backing TLC plates (Whatman PE-SIL-G) were loaded with crude extract from each isolate and run with benzene:acetone (2:1) separation mixture (Ahmad *et al.*, 2011). Once individual bands were received, each were cut out, scraped and placed into an ampule, prior to an extraction once more with HPLC grade 95% acetone.

6.4.6. Pigment structural elucidation

Dried crude pigment extract from strain P117 (50.2 mg) was dissolved in 10 mL DCM and stirred for 18 h. The DCM mixture was then filtered, and the remaining solids were collected with acetone that was then removed by rotary evaporation. The sample was dissolved again in DCM and stirred for 2 h, solid collected,

and repeated until 3 mg of partially purified material was accumulated. The process was repeated for an extract from P102. While NMR data were collected for P117, LC-MS/MS and HPLC were carried out on both samples, revealing the presence of violacein and deoxyviolacein.

All NMR experiments were recorded on a Bruker Avance 500 spectrometer. NMR techniques included ^1H NMR, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC). HPLC was carried out using a Waters Alliance 2695 Separations Module with Waters 2996 Photodiode Array Detector, and a bondapak C_{18} column (300 mm $\times$ 3.9 mm, 125 Å, 15–20 mm). The mobile phase consisted of 0.75% trifluoroacetic acid (HPLC grade) in water (solvent A) and methanol (HPLC grade) (solvent B). The gradient was set as 0–10 min (20% B), 10–20 min (20–100% B), 20–40 min (100% B), and 50–60 min (100%–20% B) using a flow rate of 1 mL/min. Injection volume was 50 μL , detection wavelength of 254 nm UV trace with full 200–600 nm spectrum recorded.

A Bruker Compact Q-TOF instrument with an electrospray ionization source was used for LC-MS analysis. Separation was achieved through an Intensity Solo C_{18} , 100 mm $\times$ 2.1 mm column with a 0.4 mL/min flow rate, eluent A was 0.1% formic acid in water, and eluent B was 100% acetonitrile. The gradient was as follows: 0–10 min (20–60% B), 10–20 min (60% B), 20–25 min (60–20% B), and 25–30 min (20% B). MS was acquired using parameters: capillary voltage 3.5 kV; gas temperature 200°C; drying gas, 3.0 L/min and nebulizer pressure of 0.3 bar; injection volume was 10 μL . Data was acquired with a mass scan range of 50–1200 m/z to observe fragmentation pattern. An extracted ion chromatogram was taken for analysis at 342 $m/z \pm 1$ for violacein and 326 $m/z \pm 1$ for deoxyviolacein, peaks were found at 7.8 and 9.6 min, respectively.

6.5. Results and Discussion

6.5.1. Violacein producing organisms in Lake Winnipeg

The bacterial community from Lake Winnipeg shorelines was investigated in 2017 for its composition of aerobic pigmented bacteria. Two isolates were initially found to be internally pigmented violet, strain P102 (Fig. 6.2A and B) and P117 (Fig. 6.2C and D), due to similar compositions of extractable pigments (Fig. 6.2E–G). On BG11 medium, P102 represented only 0.001% of culturable bacteria at Site 2, which had a total count of 1.80×10^5 CFU/mL. Of the 4.27×10^3 CFU/mL on PA at Site 4, P117 made up 0.023%. While these low numbers most likely represented the small population of violet aerobes within the bacterial community, they may also have been the result of non-selective cultivation techniques. To identify phylogenetic relatives and

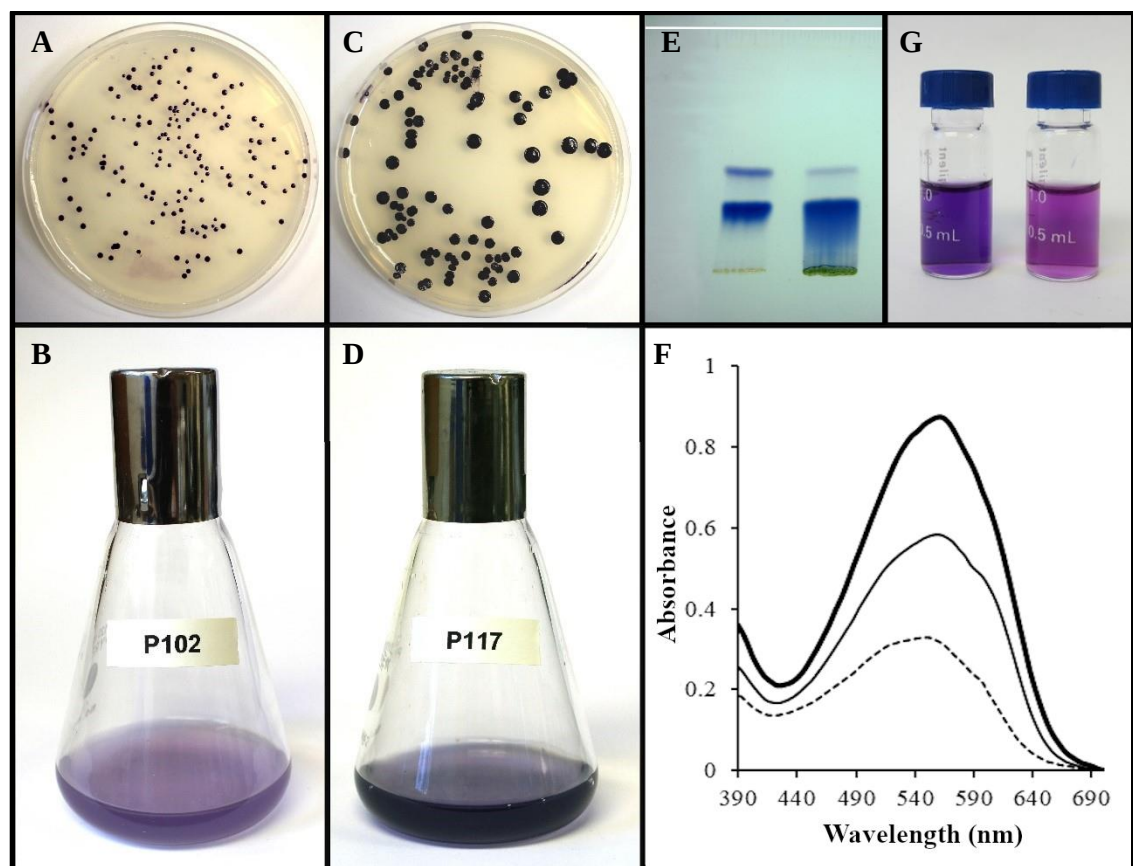


Fig. 6.2. New violet bacteria enriched from Lake Winnipeg, and their isolation of pigments via TLC. (A) Strain P102 on agarized RO, and (B) within liquid medium after 7d incubation. (C) Strain P117 on plate, and (D) within liquid RO medium. (E) Acetone extracts run on a TLC plate with 2:1 benzene:acetone, revealed two bands from either stain. (F) The absorption spectrum of crude acetone extract, —; Band 1, - -; and Band 2, - - -; were identical between isolates: Band 1 had a 562 nm peak, and Band 2 had a 552 nm peak. (G) Corroborating divergent violet hue of each excised band in acetone.

better assess their growth requirements, the 16S rDNA of strains P102 and P117 were sequenced. P102 had 99.30% sequence similarity to *Janthinobacterium lividum*, DSM 1522^T, and P117, 99.17% to *Massilia violaceinigra*, B2^T (Table 6.1). Although both relatives *J. lividum* and *M. violaceinigra* were reported as purple (Inniss and Mayfield, 1979; Choi *et al.*, 2015b), no specific enrichment medium had been designed for isolation and optimal growth. To date, violacein producing bacteria have been cultivated on Nutrient Agar, or slightly modified variations containing complex organics that also promote the development of numerous other bacteria (Moss *et al.*, 1978; Avguštin *et al.*, 2013; Choi *et al.*, 2015b). Rather than yeast extract or peptone, diatom extract has been used in media for the isolation of *J. lividum* from another large and temporally icecovered fresh water Lake Baikal in Russia (Bashenkhaeva and Zakharova, 2017). However, until an effective selective medium is designed, violet aerobic bacteria will be cultivated from the environment with a cohort of all other heterotrophic bacteria capable of growth on complex media, and therefore be isolated in a competitive circumstance.

To examine this population within the lake without the need of cultivation, we examined 16S V4 rRNA gene sequences extracted from samples collected in 2017 (Chapters 2,3). Curation of annotated genera revealed that violacein producing *Duganella*, *Janthinobacterium*, *Chromobacterium*, as well as other *Burkholderiaceae* likely including *Collimonas* and *Massilia* made up to 5.514% and 6.258% of all bacterial and archaeal genes present in environmental water and sediments, respectively, at Sites 1 through 3. No genes from *Pseudoalteromonas* or *Microbulbifer* were detected, potentially because both *Pseudoalteromonas luteoviolacea* and *Microbulbifer hydrolyticus* possess absolute requirements for NaCl, which is absent in Lake Winnipeg. It is important to mention that these values included all organisms related to purple pigment producers, as resolution of species within genera was not possible with the sequencing technique, and also because of their very low presence. Because some genera like *Janthinobacterium* were described to include representatives that did not produce violacein, for example *J. agaricidamnorum* (Lincoln *et al.*, 1999), these numbers may be an overestimate. However, among species tested, many produced violacein only under some specific conditions; therefore, the pigment-free species mentioned above may also produce violacein, but have simply not been exhaustively examined. Considering both estimates above as the upper and lower possibilities, violacein producers may represent between 0.001% and 6.258% of active bacteria in the lake during colder months.

Table 6.1. Violacein containing bacteria isolated from Lake Winnipeg.

Year	Ssn	Site	#	Colour	Colony Consistency	Closest Related Type Species	Bp	Similarity*
2017	Sp	S2	102	Violet	Smooth	<i>Janthinobacterium lividum</i> , DSM 1522	1435	99.30
2017	Fa	S4	117	Dark Purple	Hard	<i>Massilia violaceinigra</i> , DSM 19531	1441	99.17
2018	Fa	S1	16	Violet	Smooth	<i>Janthinobacterium lividum</i> , DSM 1522	1442	99.10
2018	Fa	S3	110	Dark Purple	Hard	<i>Massilia violaceinigra</i> , DSM 19531	1450	98.90
2018	Fa	S4	63	Dark Purple	Hard	<i>Massilia violaceinigra</i> , DSM 19531	1437	99.16
2019	Wi	W6	34	Violet	Smooth	<i>Janthinobacterium lividum</i> , DSM 1522	1447	98.83
2019	Wi	W6	35	Violet	Smooth	<i>Janthinobacterium lividum</i> , DSM 1522	1424	99.86
2019	Wi	W6	41	Violet	Smooth	<i>Janthinobacterium lividum</i> , DSM 1522	1430	98.68

Ssn = season, Sp = Spring, Fa = Ssn = season, Sp = Spring, Fa = Fall, Wi = Winter; Sites as seen in Fig. 6.1; # = isolated from that site; 16S rDNA sequences compared to type species in NCBI database, with base pair (Bp) length of sequenced fragment; *16S rDNA sequence similarity (%) to closest related type species. Fall, Wi = Winter; Sites as seen in Fig. 6.1; # = isolated from that site; 16S rDNA sequences compared to type species in NCBI database, with base pair (Bp) length of sequenced fragment; *16S rDNA sequence similarity (%) to closest related type species.

Comparing to two other large eutrophic freshwater lakes covered by ice for 1–2 months a year in Europe, 16S rRNA gene sequences of the genus *Janthinobacterium* have been recently found as the major contributors to the extracted bacterial communal DNA (Bullerjahn *et al.*, 2020). Samples from the ice and water phases of Lake Balaton had 14.5 and 7% of genes, respectively, to represent this genus, and in Lake Ferto they contributed 1.3% and 4.1%. Although *J. lividum* was cultivated from Lake Baikal (Bashenkhaeva and Zakharova, 2017), the early compendium study did not reveal any sequences of the violacein producers among the bacterial community (Bashenkhaeva *et al.*, 2015). In both Lake Baikal and under the ice of Lake Erie (Beall *et al.*, 2016), the β -*Proteobacteria* subphylum species made up a dominant portion of the 16S rRNA sequences. While neither study could determine speciation, our results and those received in the analysis of Lake Baikal suggest violacein producers and their possibly colourless relatives make up a significant proportion of the community during colder months.

In general, not many ecological investigations of natural violacein producers have been performed, and while some data from other temperate eutrophic lakes exist, Lake Winnipeg overall has had very little information regarding bacterial composition. Only results published from a few studies related to coliform counts at public beaches during the summer are known (Pip and Allegro, 2010), but practically none is available for the total bacterial community composition during any season.

Recovery of the two aerobic violet organisms P102 and P117 in 2017 and noting their cultivation during colder months stimulated additional expeditions to search for more isolates. Further enrichment attempts the following two years yielded six new strains also found during the colder seasons of spring, fall or winter (Table 6.1). Similar to P102 and P117, each produced violet pigments extractable in polar solvents with identical absorbance characteristics (Fig. 6.3). Our later discoveries encouraged more thorough examination of the two initial isolates to understand the physiology and possible role of pigmentation.

6.5.2. Pigment production under different growth conditions

In liquid culture, P102 and P117 revealed a variety of temperatures, pH and carbon sources to be either optimal for growth or stimulating for pigment production after a 7-day incubation (Table 6.2). Cells required low aeration to develop colour, growing on a shaker at 100 rpm, whereas high aeration showed inhibitory effects, when all other conditions were kept at optimum. When grown aerobically on plates, a larger range of conditions were stimulatory, including temperature and pH extremes when left for several weeks. Comparable results have been previously recorded for other violacein producers (Yang *et al.*, 2007). Strain

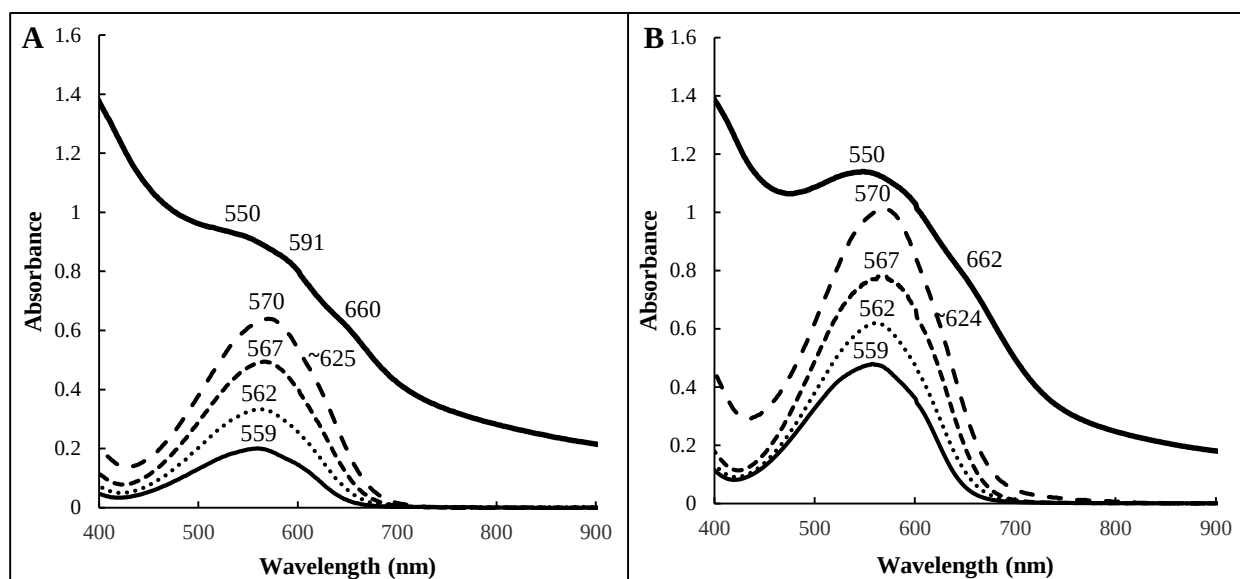


Fig. 6.3. Absorption spectrum of whole cells and extracts. (A) Strain P102, and (B) Strain P117 absorbance of intact bacterium *in vivo*, —; pigments extracted in acetone, -; 7:2 acetone:methanol, . . . ; 1:1 acetone:methanol, - - -; and methanol, - -. Maxima, and presence of shoulders are indicated (nm).

Table 6.2. Influence of conditions on growth and violacein production.

Condition			P102		P117	
Carbon source	°C	pH	Growth	Violacein	Growth	Violacein
RO	4	7	+	-	++	+
	6		++	-	+++	+
	8		+++	+	+++	++
	15		++++	+	++++	++++
	20		++++	++++	++++	++++
	25		+++	+	+++	++
	28		+	-	+	-
	37		+	-	-	-
	45		-	-	-	-
	50		-	-	-	-
RO	5	20	-	-	-	-
	5.5		-	-	+	+
	6		+++	+++	++	++
	7		++++	++++	++++	++++
	8		++++	++++	++++	++++
	9		+++	+++	+++	+++
	10		++	++	++	++
	10.5		+	+	-	-
	11		+	+	-	-
	11.5		-	-	-	-
	12		-	-	-	-
Acetate			+	+	-	-
Bactopeptone			+	+	+	++
Citrate			+	+	-	-
Ethanol			+	+	-	-
Fructose (D-)			++	+	+	+
Glucose (D-)			++	+	++	+++
Glutamate (L-)	20	7	++	++	+	+++
Lactose (α -)			+	+	-	-
Pyruvate			++	++	+	++
Succinate			+	+++	+	+++
Yeast Extract			+++	++++	++	++++
Sucrose			++	+	+	+
Acetate			+	+	-	-
Bactopeptone			+	+	+	+
Citrate			+	+	-	-
Ethanol			+	+	-	-
Fructose (D-)			+	+	+	+
Glucose (D-)			++	+	++	+++
Glutamate (L-)	25	7	+	+	+	+++
Lactose (α -)			++	+	-	-
Pyruvate			+	+++	-	-
Succinate			+	++	+	+
Yeast Extract			+++	+	+++	++
Sucrose			+	+	-	-

Whole cell OD and violacein absorption were measured at 660nm and 560nm, respectively, after 7 day incubations; Absorbance recorded as 0-0.05, - ; 0.05-0.25, +; 0.25-0.50, ++; 0.50-0.75, +++; 0.75<, +++++.

P102 grew optimally at 20°C, pH 8 in RO, while also producing the highest amounts of pigment. P117 behaved similarly in RO medium, with optimal growth at 15°C and pH 8, which were also optimal for the violet pigment accumulation. When challenged against suboptimal temperature, pH or carbon source, both strains grew very poorly and were less pigmented. As suggested in section 6.5.1, the synthesis of violet pigments may require specific conditions for initiation. Our results indeed confirmed, that optimal growth temperatures were required for the maximal pigment production. This can be compared to other small molecule synthesis, where some are produced under optimal conditions while others are produced when the organism in question is stressed (Soliev *et al.*, 2011).

To expand analysis of the link between optimal growth and pigmentation, a variety of organic carbon sources were added to the medium at either 20 or 25°C (Table 6.2). Under all circumstances, when the strains grew optimally, they were most intensely pigmented. When they grew sub-optimally due to unfavorable conditions, proportionally less colour was developed.

The optimal growth at slight alkalinity of both strains matched our measurements from Lake Winnipeg during sampling, pH 8.6–8.9, suggesting adaptation to the alkaline environment. Furthermore, both strains could not grow anaerobically which also corresponds well with the place of isolation, as the shallow South Basin of Lake Winnipeg is generally considered polymictic and predominantly aerated from the top to the bottom (MWSD, 2011). The freshwater character of the lake was further represented by the inability of our strains to grow efficiently in the presence of NaCl. When 2% NaCl was supplemented to RO at pH 8, both P102 and P117 grew poorly and only 30 or 35% of pigments of that measured in cultures grown without salt were observed. These results are strikingly different from those reported for strains of *Janthinobacterium*, which required NaCl for growth (Avguštin *et al.*, 2013). On the other hand, isolates related to *M. violaceinigra* based on 16S rRNA sequences were more similar, as this type species was also sensitive to NaCl (Wang *et al.*, 2018). Although P102 and P117 produce pigments maximally at 15 to 20°C with similar absorbance characteristics to violacein in cells of *Chromobacterium lividum* (Inniss and Mayfield, 1979), their optimal growth temperatures were lower (Fig. 6.4 and Table 6.2). According to currently defined temperature ranges of growth preferences (Wiegel, 1990), P117 behaves as a psychrophile with an optimum at 15°C, whereas P102 can be considered a psychrotroph, preferring 20°C, but tolerating temperatures as low as 6°C and as high as 37°C.

6.5.3. Growth rate vs. pigment production

In both P102 and P117, regardless of growth temperature, pigment was synthesized only once cultures reached stationary phase (Fig. 6.4), with the highest concentration after 168 h. Strain P102 grew optimally at both 15 and 20°C, but only 30% as efficiently at 28°C (Fig. 6.4A). Although P117 reached the same OD plateau in stationary phase at both 15 and 20°C, occurring after 58 h, the OD increased more rapidly at 15°C, evident after 24 h (Fig. 6.4B). P117 also reached stationary phase at 28°C after 58 h, but its OD plateaued at ~0.7, or 50% of yield at colder incubation. After 58 h, cells had likely halted their replication, and instead began producing their intracellular pigment content between 58 and 168 h, turning the cultures violet (Fig. 6.4C and D). For strain P102, pigmentation was most prolific at 20°C after 10 days (Fig. 6.4C), but only produced <70% of that estimated in P117 in identical conditions (Fig. 6.4D). Furthermore, cultivation of P102 at 15°C did reveal pigment production, but significantly reduced, as seen in the 7 day experiments at each temperature (Table 6.2). In comparison, P117 produced its highest amount of pigment at 15°C, and it grew to stationary phase the fastest. When cultivated at 25°C, both strain's OD plateaued at 30% of that at optimal growth, and cells remained colourless. These results show the requirement of lag time for the expression of pigment production, and suggest that close phylogenetic relatives may need further analysis to determine their pigment production potential.

6.5.4. Pigment extraction and identification

Polar organic solvents were required to extract the dark violet pigment from cells of P102 and P117. Methanol, acetone and ethanol were all effective solvents for the extraction (Fig. 6.3A and B). Use of non-polar benzene and chloroform failed to work independently, suggesting the highly polar nature of the violet compounds. MeOH extracts had an absorbance peak at 570 nm, and upon dilution with acetone, slowly blue shifted the maximum to 559 nm. Similar maxima were previously reported for violacein, mildly red or blue-shifted depending on solvent (Rettori and Durán, 1998; Ahmad *et al.*, 2011). When extracted in acetone, the violet solution had pH 7.5. While the acidification had no effect on colouration or absorbance spectrum of crude extract, the addition of NaOH to alkaline conditions over pH 13 resulted in a change of hue to blue (Fig. 6.5). Furthermore, the absorbance spectra of the blue solutions were comparable to previously observed in extracts from *C. violaceum* (Ahmad *et al.*, 2011), suggesting similar chemical arrangements.

Separation via TLC of small polar molecules produced by P102 and P117 resulted in two migrated violet coloured bands from each isolate (Fig. 6.2E). The bands from both strains had very similar colouration and migration distances to one another. R_f values of bands 1 and 2 from P102 were 0.47 and 0.35 for its upper

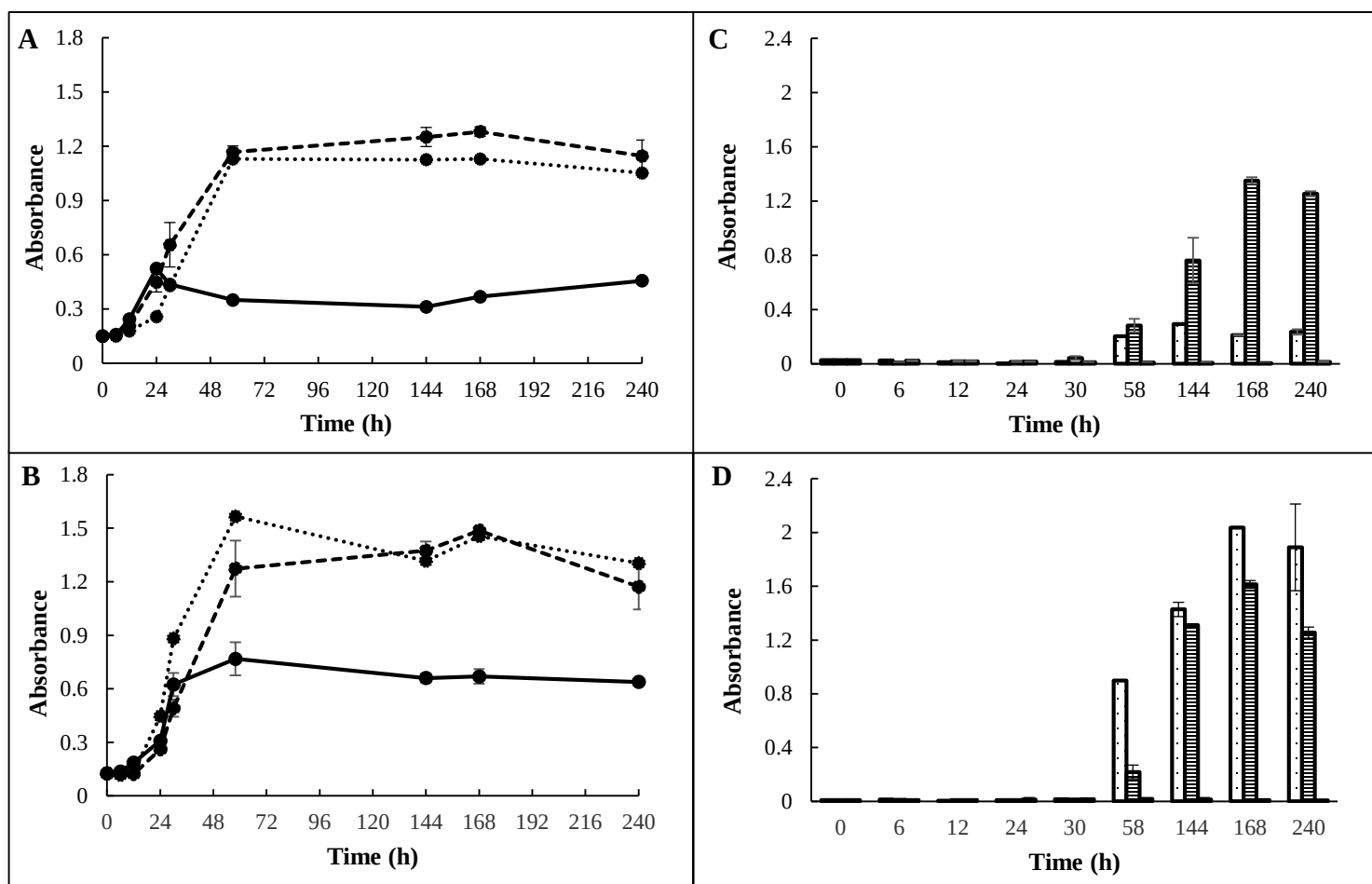


Fig. 6.4. Growth curves, and pigment production measured at varied incubation temperatures. (A, C) Strains P102 and P117, respectively, had optical density of whole cells, measured at 660nm over 10 days at 15°C, ●●●; 20°C, ---; and 28°C, —. **(B, D)** Strains P102 and P117, respectively, violacein was quantified at 560nm, by extraction in 0.5 mL 100% acetone from cell pellet of 1 mL culture during each time point, at 15°C, □; 20°C, ◻; and 28°C, ■.

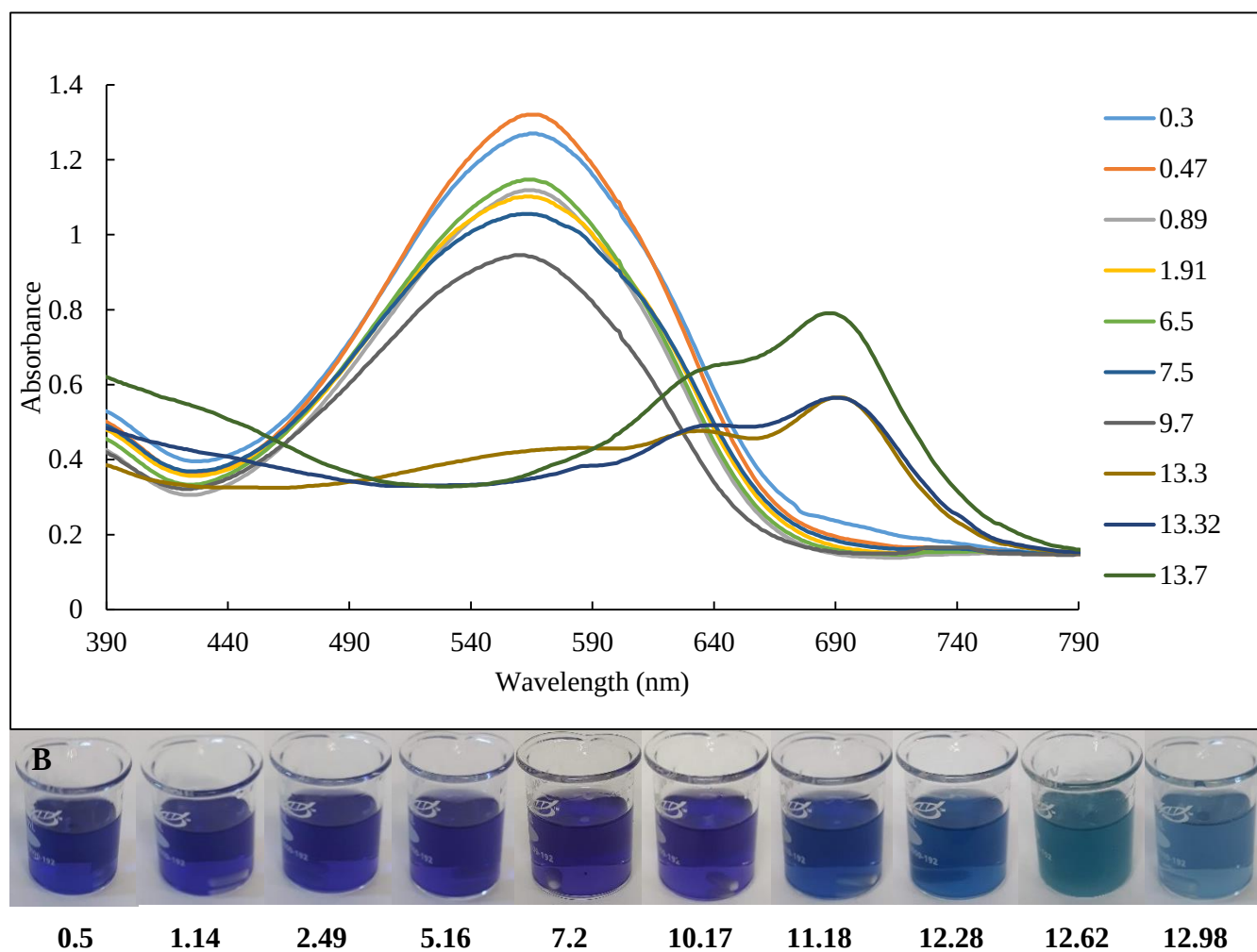


Fig. 6.5. Crude purple extract of P102 in acetone. Changing pH with addition of 2N HCl or NaOH resulted in different looking absorbance spectrum (A), and pigmentation (B). P117 extracts reacted identically to P102 (not shown).

and lower band, respectively. Extract from P117 had similar Rf values for both bands, at 0.50 and 0.36, respectively. Although similar in colour on the TLC plate, the hue of each migratory band differed after separate excision and re-extraction (Fig. 6.2G). In addition, the upper and lower bands had divergent absorbance spectra, with maxima at 517, 552, and 597 nm for the upper, and 517, 562, and 597 nm for the lower, respectively (Fig. 6.2F). A published Rf value for violacein methanol extracted from *C. violaceum* was 0.43 when run with 2:1 benzene:acetone, with an additional spot at Rf 0.50 that represented deoxyviolacein (Ahmad *et al.*, 2011). As these values were comparable to results of our study, it is likely strains P102 and P117 produced similar molecules.

Pigment structural analysis from strain P117 was completed via ¹H NMR, HSQC, and HMBC (Figs. 6.6-6.9), and the major pigment produced was confirmed to be violacein (Fig. 6.10A and Fig. 6.11). The coloured compound from P102 was identically examined, and the results were alike (Table 6.3). Furthermore, a less prevalent species of violacein, deoxyviolacein, was also present in both samples (Fig. 6.10B). In unison, the deoxyviolacein was less concentrated than violacein in both species (Fig. 6.2E), requiring heavier suspensions loaded from P102 to observe the representative band. The combination of variable production of violacein and deoxyviolacein, and their difference in hue (Fig. 6.2F-G) are the likely causes of divergence in colony and liquid culture colouration of the two strains. While P102 predominantly produced violacein, and was therefore lighter violet, P117 contained more deoxyviolacein, the darker pigment, and consequently was of deeper violet shade (Fig. 6.2B and D). Further comparison of pigments from P102 and P117 to commercially available violacein (>98%) extracted from *J. lividum*, SigmaV9389-1MG, via HPLC and UV trace also confirmed their alike identities (Figs. 6.12-6.6.14). LC-MS intensities and fragmentation patterns of samples from P102, P117, and commercial extract from *J. lividum* all revealed the presence of violacein (Figs. 6.15 and 6.16) and deoxyviolacein (Figs. 6.17 and 6.18). Hence, combined results of HPLC extraction, UV trace, LC-MS, and fragmentation patterns obtained in our work coincided well with violacein and deoxyviolacein features reported from *J. lividum* (Hoshino *et al.*, 1987).

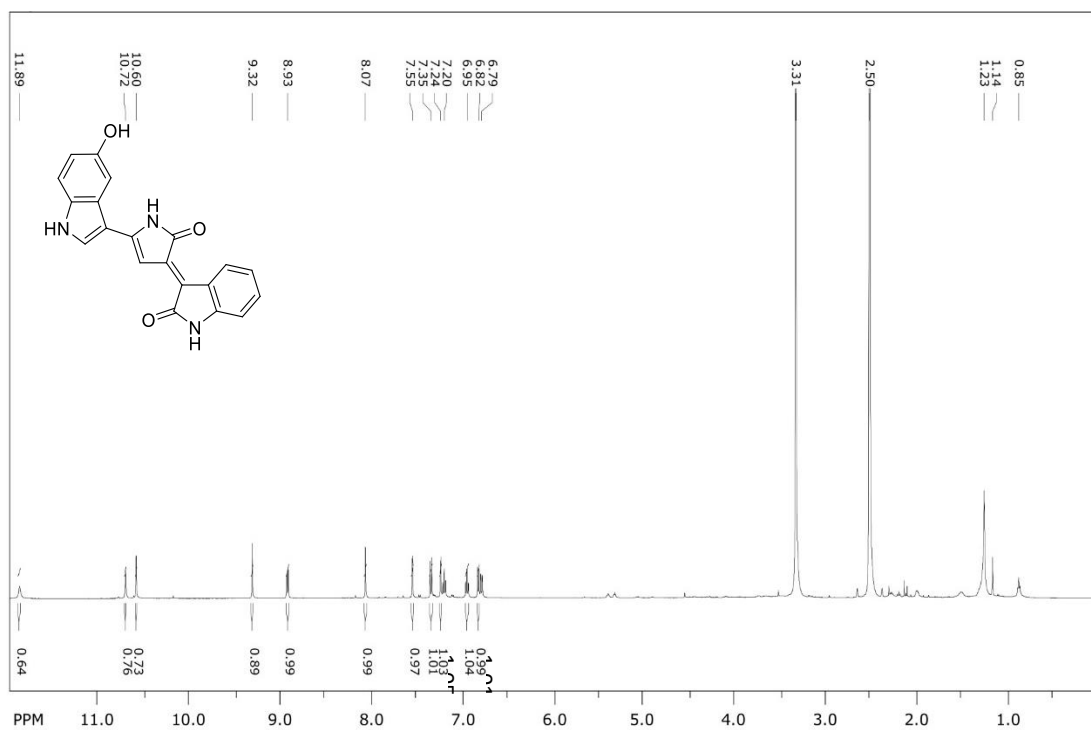


Fig. 6.6. ¹HNMR of P117 violacein extract acquired from 0-12 ppm; ((CD₃)₂SO, 500 MHz, ppm).

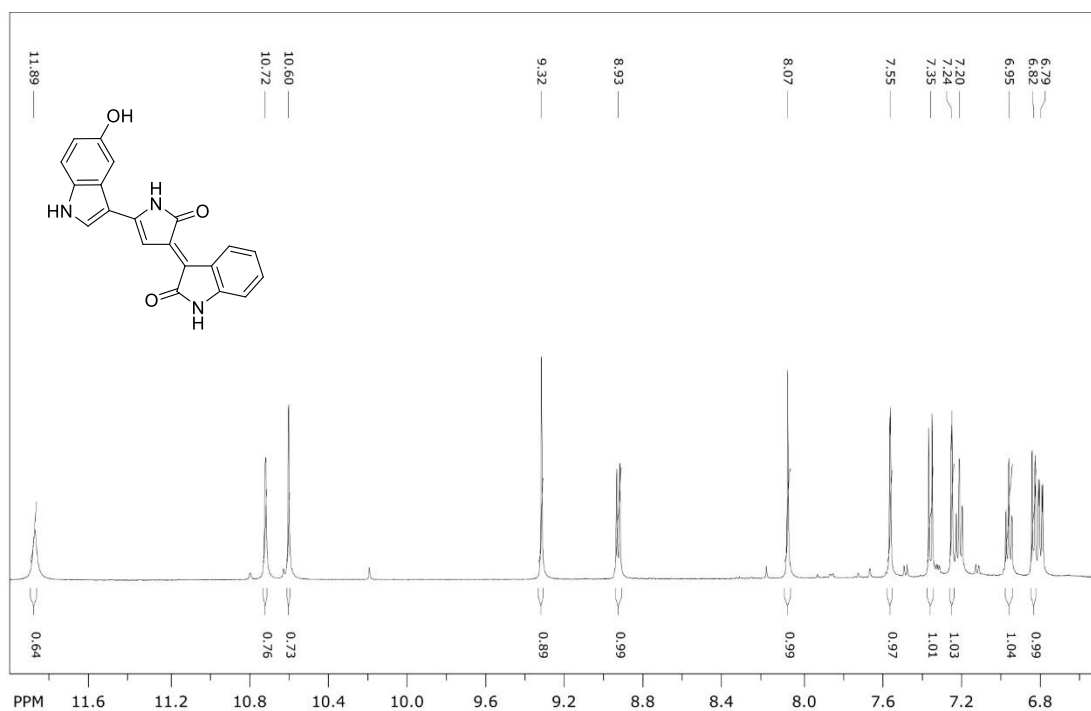


Fig. 6.7. Expanded ¹HNMR of violacein acquired from P117 between 6.5-12 ppm; ((CD₃)₂SO, 500 MHz, ppm).

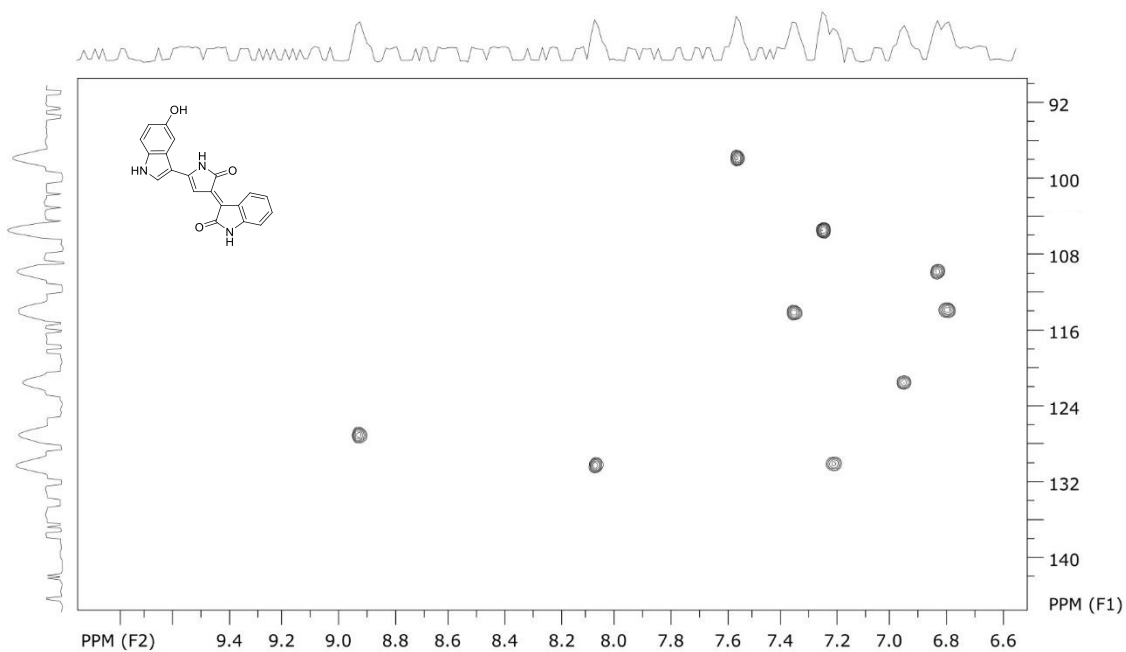


Fig. 6.8. HSQC of violacein acquired from P117; ((CD₃)₂SO, 500 MHz, ppm).

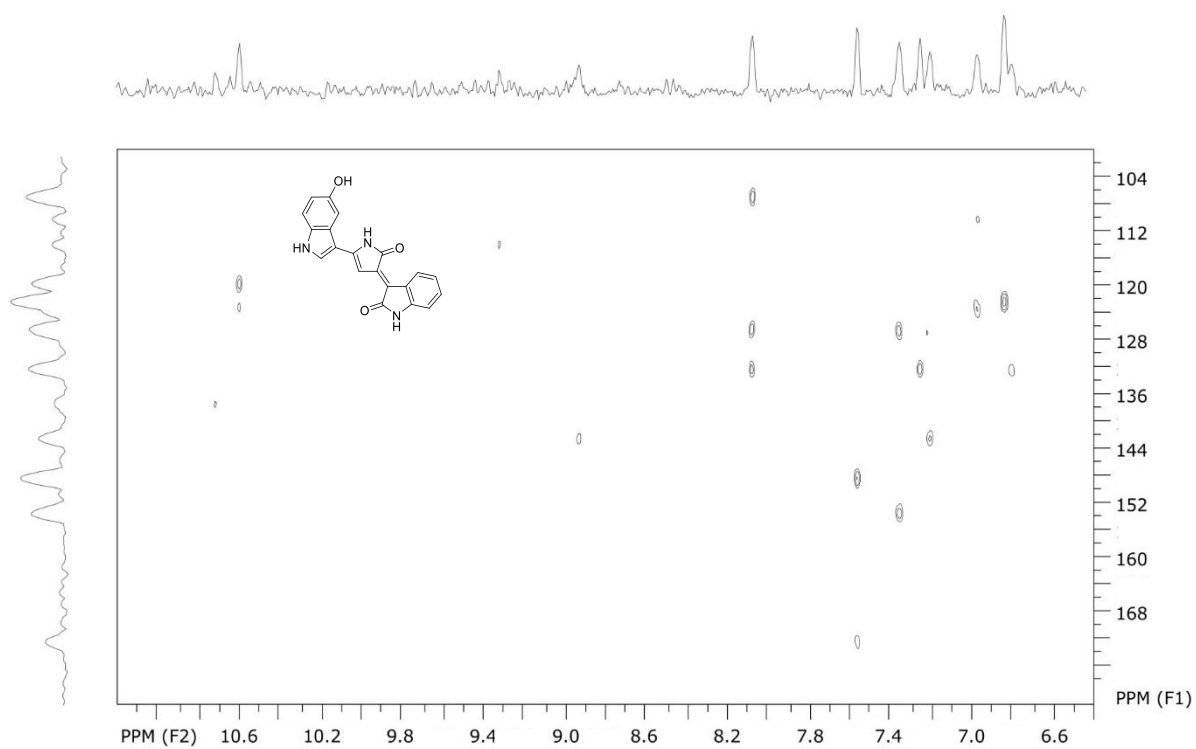


Fig. 6.9. HMBC of violacein acquired from P117; ((CD₃)₂SO, 500 MHz, ppm).

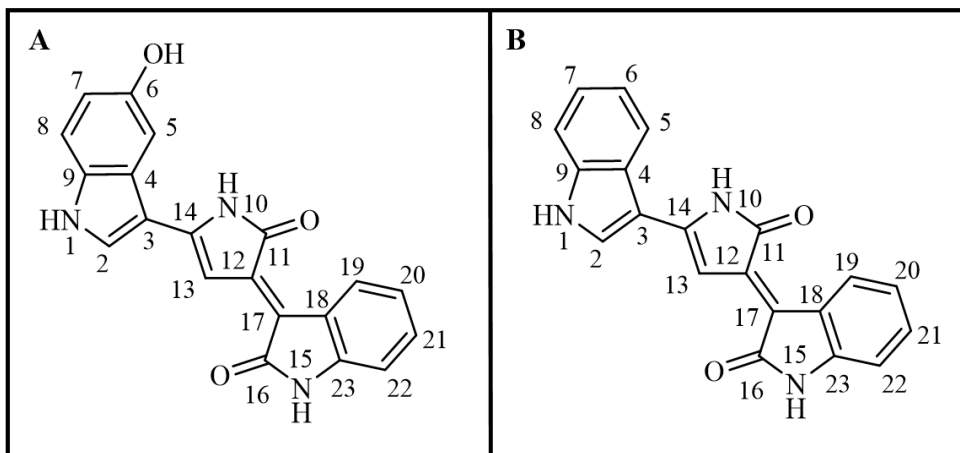


Fig. 6.10. Structure of violacein (**A**) and deoxyviolacein (**B**), purified from strains P102 and P117.

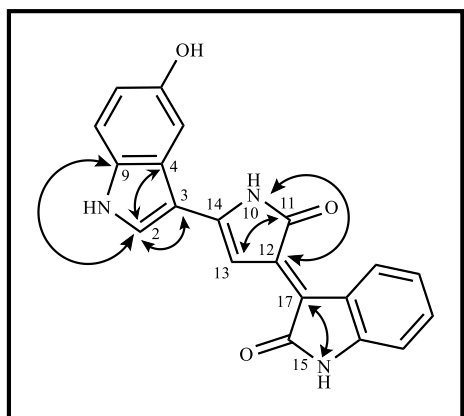


Fig. 6.11. Key proton-carbon connectivities extracted from HMBC data (Fig. 6.9).

Table 6.3. Literature (Hoshino *et al.*, 1987) and observed ^1H and ^{13}C NMR data of violacein compared to experimental data in DMSO-d6.

NUMBER	Literature ^1H NMR ¹	Literature ^{13}C NMR	Sample ^1H NMR	Sample ^{13}C NMR
1	11.89(br.s)	-	11.90 (br.s)	-
2	8.07 (d, j = 2.9)	129.5 (d)	8.07 (s)	129.9
3	-	105.8 (s)	-	106.3
4	-	125.5 (s)	-	126.1
5	7.24 (d, j = 2.2)	104.5 (d)	7.24 (d, J = 2.1)	105.1
6	9.35 (br.s)	152.9 (s)	9.32 (s)	153.4
7	6.78 (dd, j = 9.2, 2.2)	113.2 (d)	6.79 (m)*'	113.5
8	7.35 (d, J= 9.2)	113.4 (d)	7.34 (d, J = 8.7)	-
9	-	131.5 (s)	-	132.1
10	10.74 (br.s)	-	10.72 (s)	-
11	-	171.5 (s)	-	172.1
12	-	136.9 (s)	-	137.5
13	7.55 (d, j $\approx$ 1)	96.9 (d)	7.55 (d, J = 1.8)	97.4
14	-	147.5 (s)	-	148.1
15	10.64 (br.s)	-	10.60 (s)	-
16	-	170.1 (s)	-	-
17	-	118.7 (s)	-	119.2
18	-	122.3 (s)	-	122.9
19	8.93 (dd, J = 1.8, 7.8)	126.3 (d)	8.93 (d, J = 7.8)	126.9
20	6.95 (dt, J = 1.8, 7.8)	120.8 (d)	6.95 (t, J = 7.7)	121.2
21	7.20 (dt, J = 1.8, 7.8)	129.3 (d)	7.20 (m)*''	130.3
22	6.82 (dd, J = 1.8, 7.8)	108.9 (d)	6.82 (d, J = 7.7)	109.5
23	-	141.8 (s)	-	142.3

Spectra of ^1H and ^{13}C NMR were measured at 500 MHz, the chemical shifts of these spectra are shown in δ (ppm). Parenthesis contained the multiplicity of signal and the coupling constants (Hz). The ^{13}C data was derived from a combination of HSQC and HMBC spectra. *' Appears as a doublet of doublets. *'' Appears as a doublet of triplets.

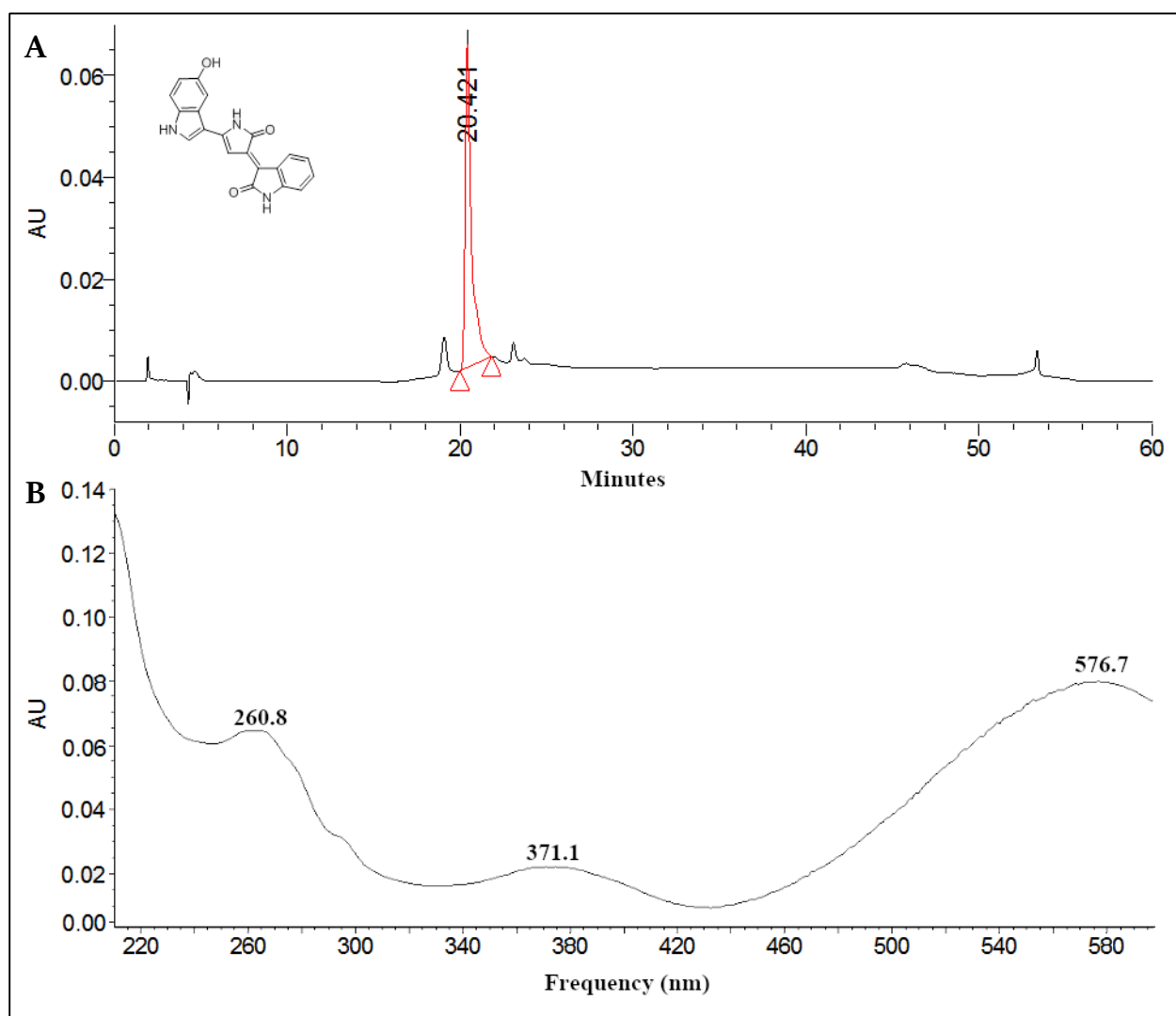


Fig. 6.12. Commercial violacein sample. (A) HPLC data of commercial sample of violacein; Wavelength 254 nm. **(B)** UV trace of commercial violacein at 20.6 minutes.

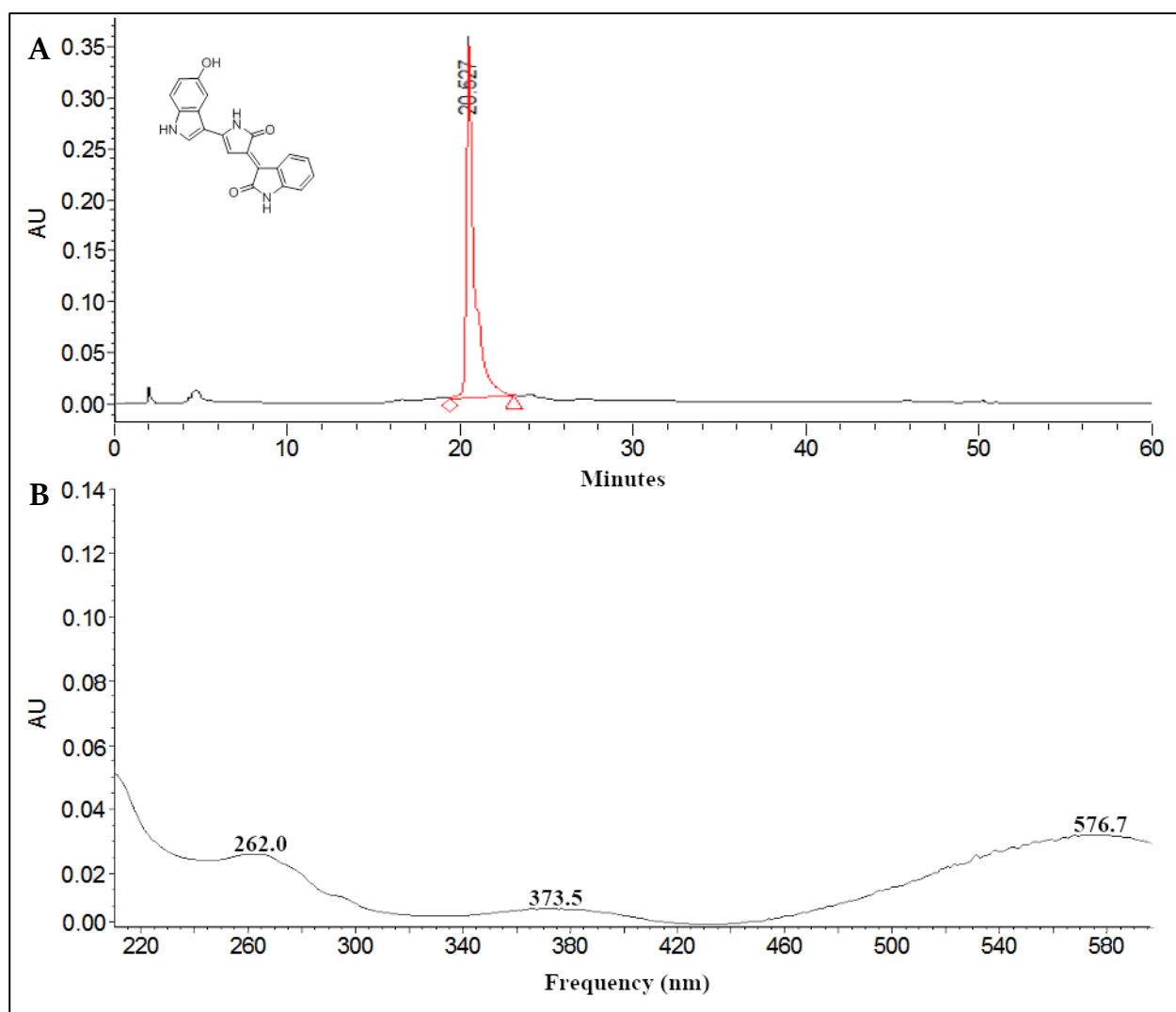


Fig. 6.13. Violacein purified from P117. (A) HPLC purified violacein extract from sample from P117; Wavelength 254 nm. **(B)** Purified violacein UV trace from P117 at 20.5 minutes.

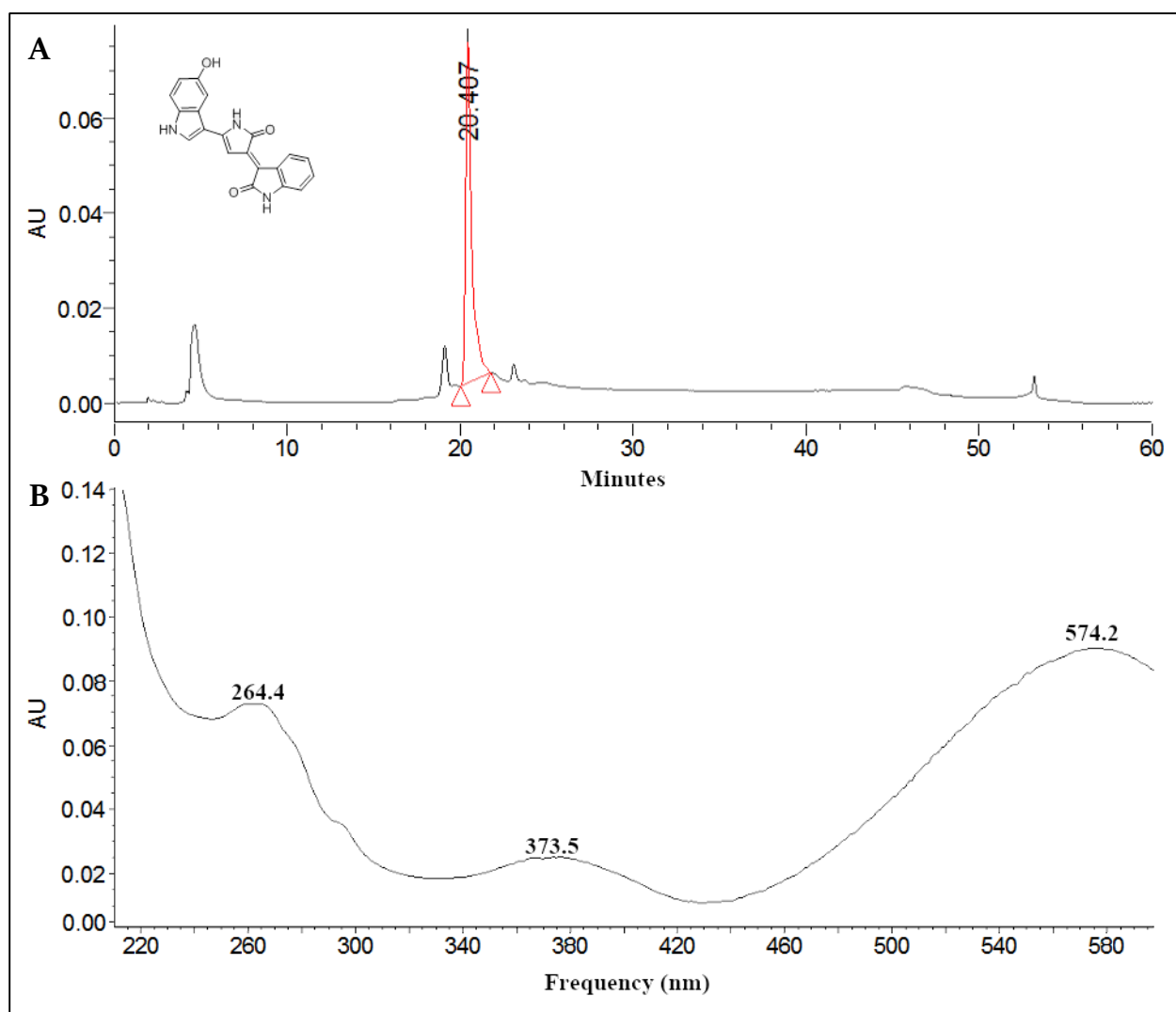


Fig. 6.14. Violacein purified from P102. (A) HPLC purified violacein extract from sample from P102; Wavelength 254 nm. (B) Purified violacein UV trace from P102 at 20.5 minutes.

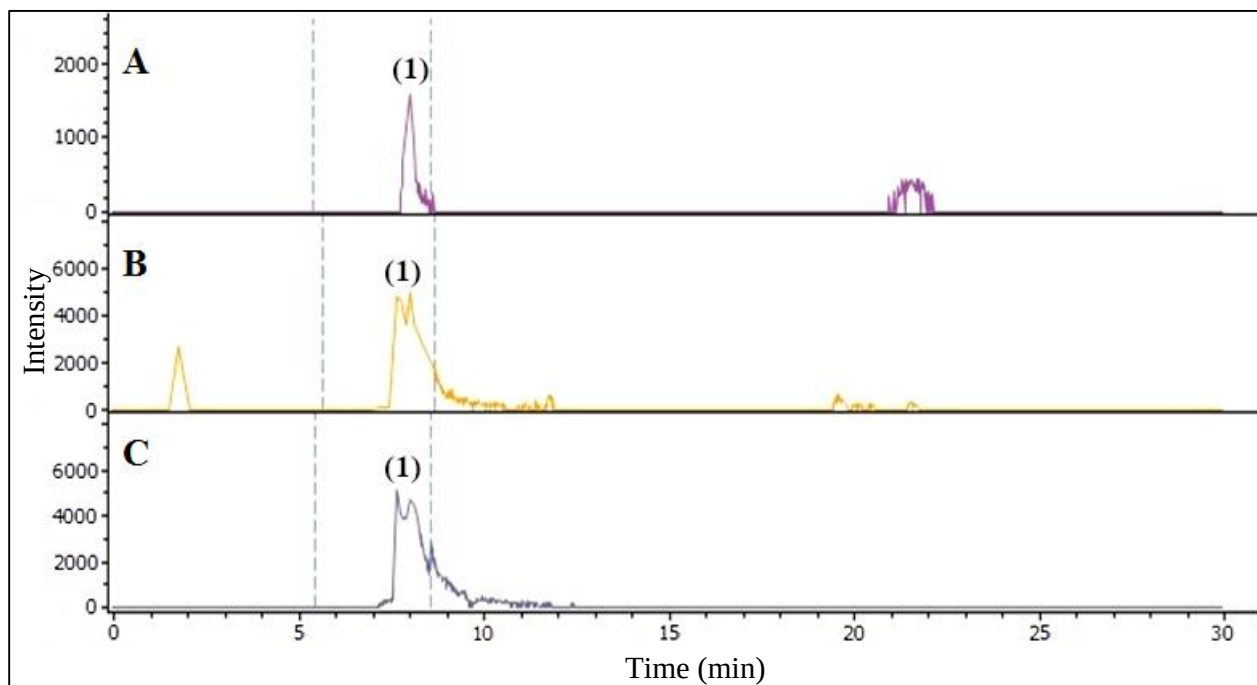


Fig. 6.15. LC-MS of Violacein (1) eluted after 7.8 minutes. P117 (A), P102 (B) and a violacein standard (C), respectively, selected for 432 ± 1 m/z. Intensity reported as Arbitrary Units (AU).

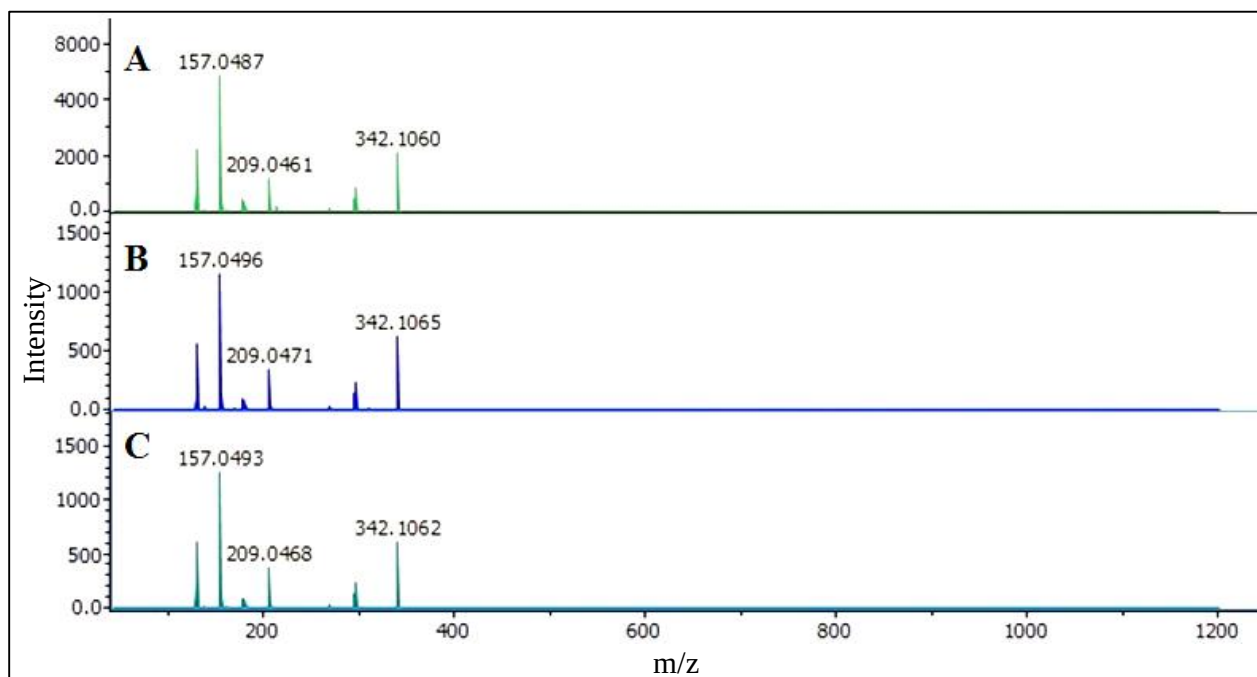


Fig. 6.16. Fragmentation pattern of violacein peak at 7.8 minutes. Samples included P117 (A), P102 (B) and a violacein standard (C) respectively. Intensity reported as Arbitrary Units (AU).

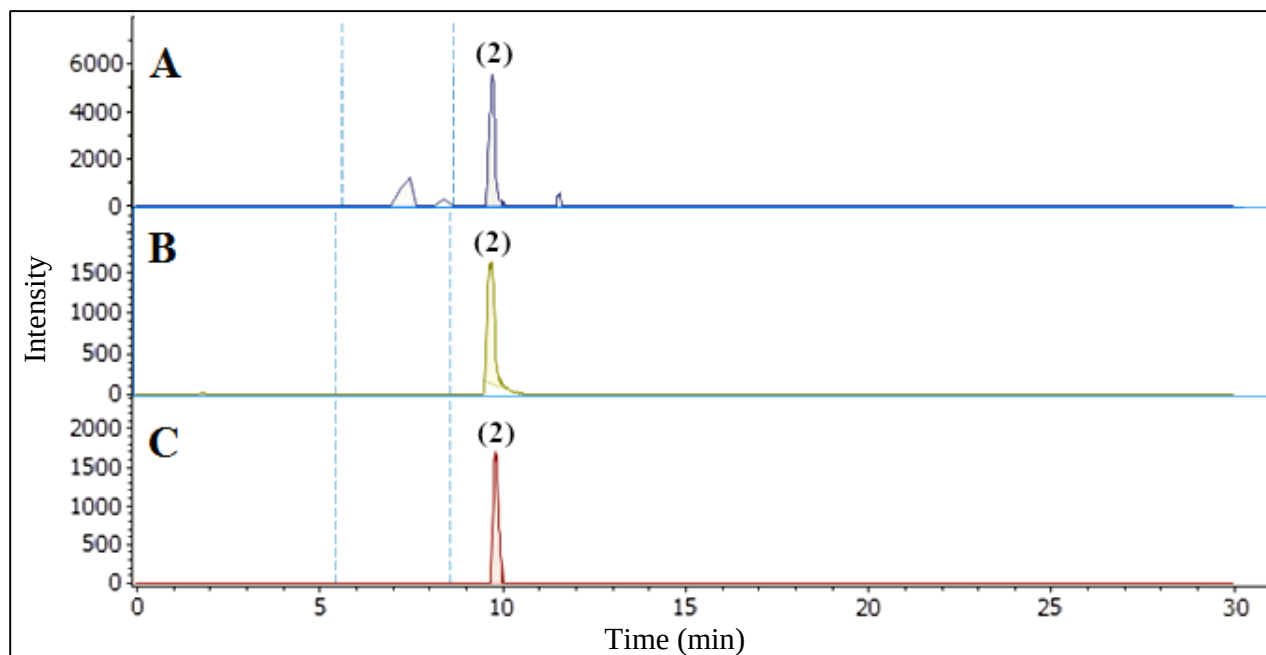


Fig. 6.17. LC-MS of deoxyviolacein (**2**) eluted after 9.7 minutes. P117 (**A**), P102 (**B**) and a violacein standard (**C**), respectively, selected for 426 ± 1 m/z. Intensity reported as Arbitrary Units (AU).

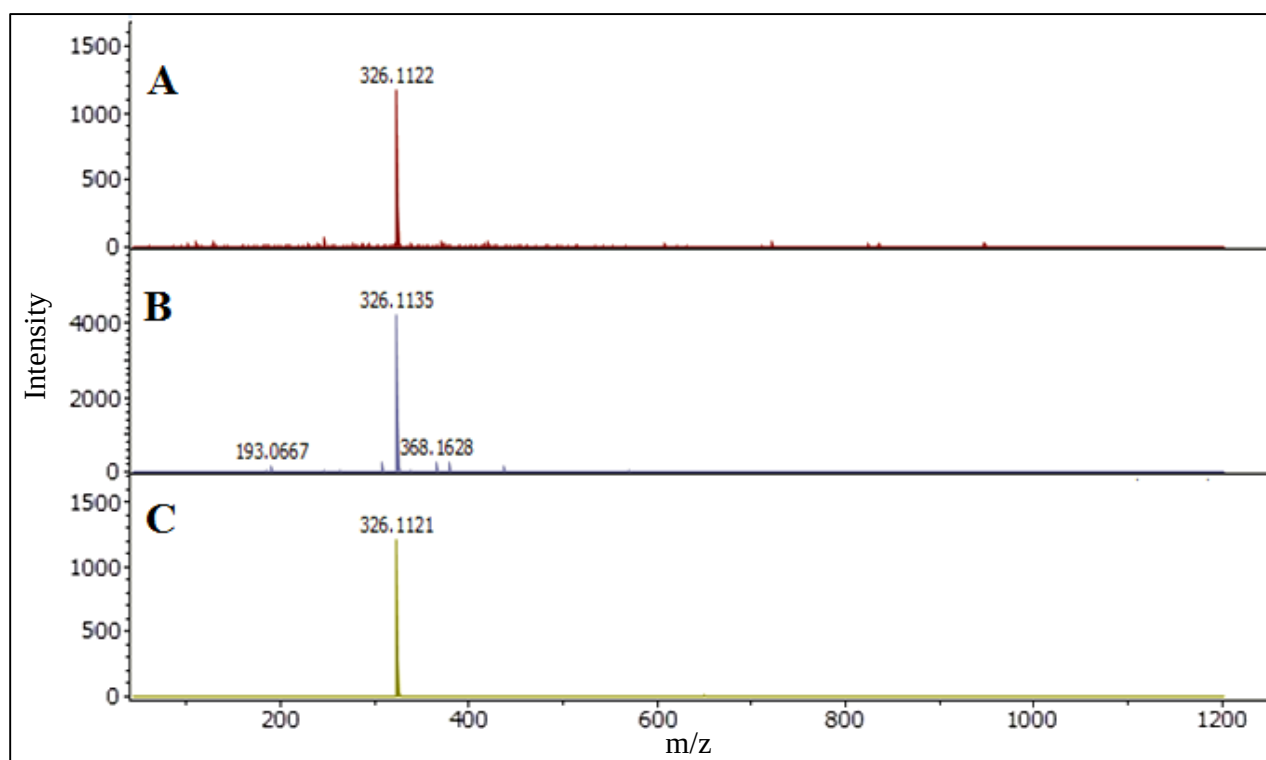


Fig. 6.18. Fragmentation pattern of deoxyviolacein peak at 9.7 minutes. Samples included P117 (**A**), P102 (**B**) and a violacein standard (**C**), respectively. Intensity reported as Arbitrary Units (AU).

In summary, among the aerobic heterotrophic bacterial community members in Lake Winnipeg, several isolates phylogenetically related to genera *Janthinobacterium* and *Massilia* were found to produce violacein. Representative strain P102 was one of five isolates that grouped together (Fig. 6.19), with 98.8–99.30% 16S rDNA sequence similarity to *J. lividum* DSM 1522 (Table 6.1). In addition, P117 represented another cluster of three tightly grouped strains that were 98.90–99.17% similar to *M. violaceinigra*. The recovery of these strains over 3 years across a vast waterbody (Fig. 6.1), and their sequence similarity to one another, provides confirmation of their consistent presence in the lake. Both carbon source and pH affected the growth of strains, whereas incubation at optimal growth conditions was required for violacein production (Table 6.2). Temperature regulated the pigment synthesis, while 15–20°C was preferred, above 25°C drastically decreased the process. Since the 16S rRNA gene sequence similarity of some isolates to known type species was near 97% and divergent phenotypes were present, future identification may confirm their novel taxonomic rank. Although the counts in Lake Winnipeg were low, between 0.001% of the culturable population and up to 6.258% of the 16S V4 rDNA sequenced sediment community, their constant detection in cold waters may suggest a larger, more important niche than previously considered.

6.6. Acknowledgements

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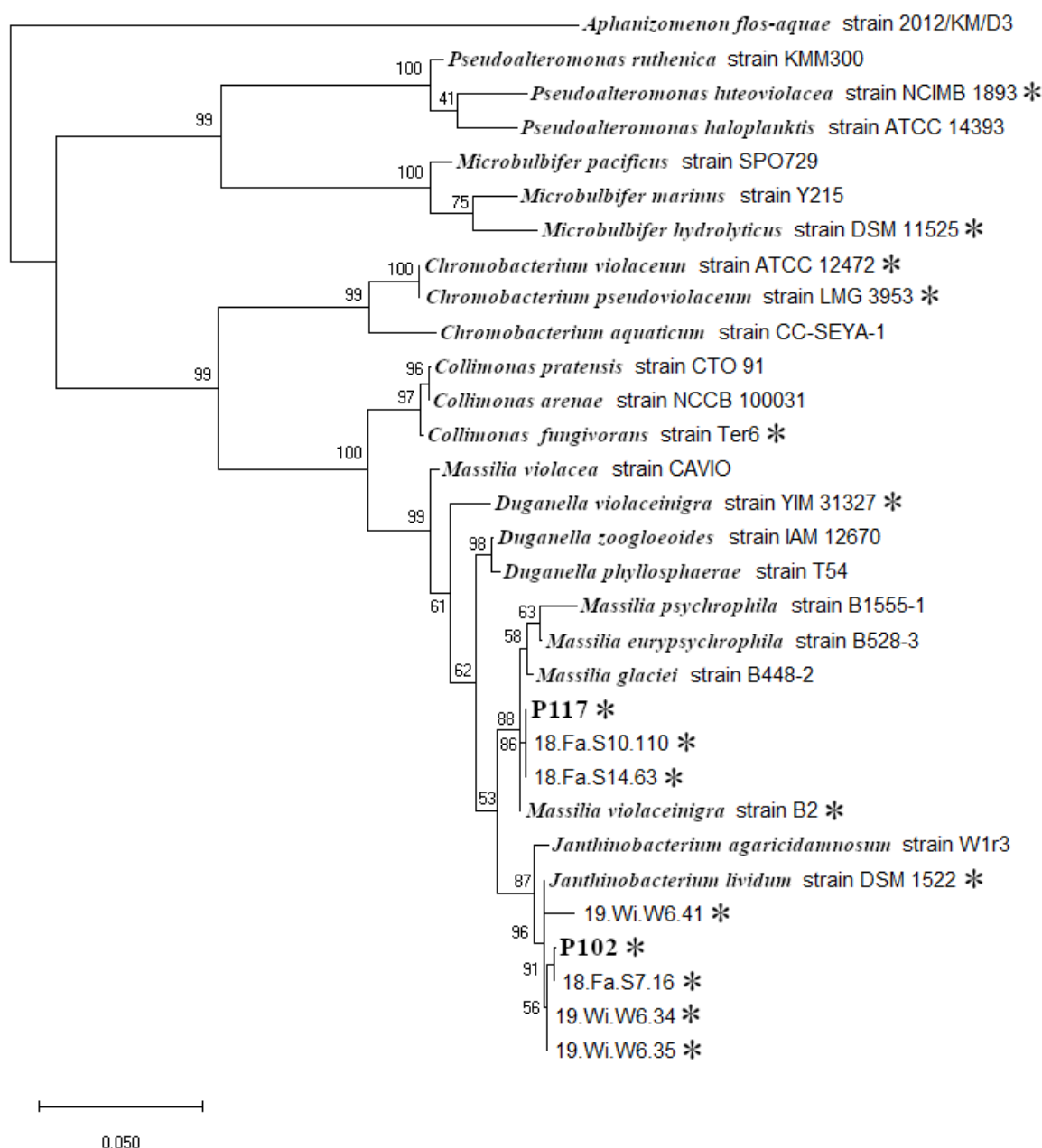


Fig. 6.19. Phylogenetic tree of 16S rRNA gene sequences of isolates among closest related type species.

The tree with the highest log maximum likelihood (6728.94) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, shown with bar. The analysis involved 31 nucleotide sequences, including type species procured from NCBI database. All positions containing gaps and missing data were eliminated. There were a total of 1282 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Species capable of violacein production are marked with an asterisk symbol.

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7. Chapter 7.
Conclusions and future perspectives

7.1. Concluding Remarks

Prior to our work, there was a knowledge gap about the microbial composition of Lake Winnipeg. The study of both limnetic and littoral zones seasonally over 4 years provided a thorough assessment of bacteria, and allowed for the correlation of environmental factors to their fluctuating numbers. We found that the lakes large yet well-mixed nature supported the establishment of a relatively stable community of *Actinobacteriota*, *Proteobacteria*, *Bacteroidota*, *Cyanobacteria*, *Planctomycetota*, *Chloroflexi*, and some other minor in presence phyla, that had reciprocal seasonal changes, returning to similar repetitive compositions each season. While limnetic and littoral zones had individual unique taxa, they additionally supported recurring levels of culturable heterotrophs, which were predominantly affected by temperature similar to AOP. Peaking at 4.23×10^6 and 7.69×10^4 CFU/mL in littoral sands and waters, respectively, mid-summer, heterotrophic cells outnumbered AOP, which were found up to 9.37×10^3 CFU/mL, suggesting high nutrient content which has been consistent with the data collected from government sources.

AnAnP were practically absent in the lake's water due to the presence of oxygen, whereas AAP were found as 19.1 and 4.7% of total copiotrophs, or 3.9 and 4.9% of oligotrophs, decreasing in counts till autumn each year. Trends were similar based also on culture-independent sequencing calculations, showing they made up 1.49 and 1.02% of the littoral and limnetic communities during the spring, also decreasing till winter. Maximal available daylight hours around Lake Winnipeg preceded water temperature maximum, which likely related to its immense size. However, this finding was shown to have unique correlations to AAP, as they directly followed light availability rather than temperature. Such a discovery was important as it directly related their numbers to the physiological function of photosynthesis, revealing it to be essential to their natural lifecycle. Moreover, bulk AAP numbers were unlinked to AOP which followed temperature, also marking it as a key differentiation, since Chl containing phototrophs have been often found in similar conditions. Our study has provided AAP data in the largest freshwater reservoir to date, and may allow for better predictions on their numbers in other great lakes.

As the seasonal microbial communities were assessed, we learned that an invasive species, zebra mussels, *D. polymorpha*, was introduced into the habitat. The result of infestation was unclear, as in some lakes they would have a boom-bust cycle and not gain a foothold in the ecosystem, while in others such as Lake Erie, they would drastically change the foodweb and diminish fish populations in some way. These filter feeders were clearly consuming something, making it unavailable for the rest of the natural predators,

creating limnetic bottom-up famine (Strayer, 2009). Seeing as they could be selectively eating small zooplankton or microbes, but no research had proven prey selectivity, we investigated potential associations with microorganisms. A zebra mussel microbiome was described, with particular bacteria found in gut contents that are strikingly different to commensal bacteria around their other organs. Furthermore, AAP were one prevalent population in their digestive tract and nowhere else, suggesting predation. This was supported when isolated AAP were shown to elongate during stationary-phase of growth, fitting within the preferential filtrate size of zebra mussels, ~5-45 μm . In addition, we revealed for the first time that bioaccumulation occurs in these small mollusks, and the AAP digested may be collecting and transforming those metal(loid)s. Such findings provide some of the earliest predation profiles of zebra mussels, which may be useful when considering mitigation of spread to other ecosystems by potentially controlling their consumption.

The cultivation of heterotrophs was fundamental for enumerating AAP, but it also provided the unique opportunity of examining all pigmented isolates developed on serial dilution plates. Curiosity was arisen, when striking violet color was observed among aerobic heterotrophs predominant in winter months. Investigation led to the identification of violacein and deoxyviolacein producing bacteria that were psychrophiles and could be cultivated or confirmed via environmental 16S rRNA sequencing as minor members only detectable during winter months. Found under the ice, they also preferentially created pigments in the cold. While not photosynthetic as no BChl was created by these bacteria, we provided the first evidence of what kind of environments violacein producers prefer, isolating them exclusively only mid-winter.

In contrast to rare violet-pigmented aerobic heterotrophs, hundreds to thousands of other pigmented isolates produced BChl *a*, one of the major features indicative of AAP. Screening strains further, our many freezer stocks became filled with a large variety of AAP examples from Lake Winnipeg. We benefited from sampling for an extended period of time as more and more diversity of phenotypes was slowly collected. Narrowing down our collection into a select few interesting strains with some unique features, relatives of 20 distinct type species were chosen for further analysis. While seasonal enumerations were useful indicators of prevalence and importance, the physiology and phylogeny of isolates informed about selective niche. Our results suggested that strains related to *Sphingomonadaceae*, *Erythromonadaceae*, *Shingosinicellaceae*, *Methylobacteriaceae*, *Acetobacteraceae*, or *Rhodobacteraceae* all shared similar features among members of

each particular family group, with minimal differences between species. Morphology, pigmentation, light absorbance by RC-LH complexes were also family dependent, pointing towards long evolutionary decent with modification and unlikely possibility for HGT. A significant finding, as some recent literature has speculated about common HGT of photosynthesis genes (Brinkmann *et al.*, 2018), yet here we show the clear differentiation of groups and no heterogeneous mixing of traits. Regarding ecology, we demonstrated for the first time that most tested AAP produced their photosynthetic pigments only under optimal growth temperatures and not during hot or cold stress. In comparison, pH acted as an on-off switch for growth and pigment synthesis for most groups, whereas *Methylobacteriaceae* related isolates had an acidic preference for BChl *a* production and *Acetobacteraceae* strains produced BChl *a* in alkaline conditions. What's more, tested oligotrophy vs. copiotrophy showed that all AAP could grow to higher biomass and enrich cell carotenoids when supplemented with enhanced concentrated complex organics. In comparison, we confirmed that BChl was produced only when relatively diluted nutrients were supplied, suggesting regulation for photosynthesis at nutrient-deprivation.

Physiological analysis of strains continued with a look at metal transformations, identifying reduction of tellurite and selenite for all 20 species. Lake Winnipeg isolates were not as tolerant to metal(loid) oxides as the earlier tested strains collected from extreme environments (Yurkov *et al.*, 1996). However, they still maintained blackening of cultures indicative of complete oxide reduction to elemental crystalline form, suggesting the mode of action is ubiquitous in many AAP. Moreover, lake strains relating to taxonomic families that had previously demonstrated extreme tolerance (Yurkov *et al.*, 1996), also had the highest levels of tolerance in this work. Apparently, certain groups are simply better at converting metal(loid)s than others via their inherited mechanisms. As an example, extreme tolerance of 2700 µg/mL tellurite had been documented for *Erythromonas ursincola*, KR99^T, of *Sphingomonadaceae* (Maltman *et al.*, 2017), and the most tolerant Lake Winnipeg strains, GM14 and BK61 were both close relatives within that family.

To further examine AAP metal interactions, we studied their ability to chelate metal(loid)s outside cells via the production of siderophores and metallophores. Indeed, we found that some strains could create secondary metabolites capable of binding up to all 10 metal(loid) cations tested, with varying efficiency. While Fe and Zn were likely collected for their biological functionality, Te and V chelation was used as a preventative measure to inhibit uptake into cytoplasm. The wide range of metal(loid) transformations suggested that AAP

have multiple modes of acquisition, and supported their importance as active participants of biogeochemical cycle of those elements, suggesting it to be a significant part of their niche.

7.2. Future Perspectives

Overall, Lake Winnipeg study should be continued as Chapter 2 provides a good framework on how it's microbial community is structured at the beginning of the 21st century. Later investigations will show how the composition alters due to climate change, the increase or decrease of pollution or invasive species, and all other factors. One of the leading contenders as highest contributor to eutrophication of Lake Winnipeg has been the historic Winnipeg North Wastewater Treatment Plant. This facility has been in need of an upgrade during the last few decades to keep up with swelling demands of increasing population. Increasing capacity and improving nutrient removal technology, instead of simply diluting excess into the Red River, may drastically reduce the effluent that reaches the lake. Potentially, such positive changes and minimizing anthropogenic influence will slowly allow for the return of conditions back to a more oligotrophic ecosystem, as historically known (Matisoff *et al.*, 2017).

Regarding the investigations of phototrophs, AnAnP were rarely detected in this waterbody, while AOP were sporadically dispersed as blooms, whereas AAP could be found evenly throughout the lake's euphotic zones. Such trends may also be in other large freshwater reservoirs, therefore, future Great Lake analysis would benefit from taking each of these photosynthetic groups into account. Furthermore, the strong correlation of AAP with sunlight availability, should be investigated in other similar habitats. As we studied them in the largest lake so far, later observations from the Laurentian Basin or Lake Baikal will likely also allow for the individualization of daylight and temperature dynamics. Seeing as we could disassociate these two important factors in Lake Winnipeg, AAP should be assessed on a seasonal basis in any other of the "top 10 largest lakes" to see if they follow available light as a predominant feature in all ecosystems.

Stopping zebra mussel advancement into unwanted ecosystems has been incredibly important, and many governmental regulations were put in place to prevent their spread. This level of awareness made us presume that a lot about *D. polymorpha* ecology was already known. It was thus surprising to learn that scientists had not yet determined what exactly they are consuming in a foodweb. Our study in Chapter 3 suggested that bivalves have dedicated microflora and may indeed be selectively grazing on microorganisms. Future work should confirm specifics of their diet, particularly relevant to AAP consumption, and look into combating invasive species by damaging their prey or natural biome. Now that this mollusk has been

introduced and detected throughout the lake (Depew *et al.*, 2021), follow-up action should include regular monitoring to assess if they will actually persist, or maybe the soft-bottomed nature of the habitat will prevent any lasting incursion. In addition, many other bivalves and crustacea are indigenous to Lake Winnipeg, and now that we know the microbial community, a great deal of analysis can go towards determining the prey of natural filter feeders and grazers. Tangentially, the bacterivorous protozoa and zooplankton could also be studied to define what their microbial preferences are, seeing as AAP have been selectively consumed by these organisms as well (Garcia-Chaves *et al.*, 2015).

Many of the AAP thoroughly analyzed in Chapter 4 only require minor additional experimentation to be fully taxonomically described as novel species. I plan on sequencing of their chromosomes in the near future to formally complete the polyphasic analysis, which will also enrich the overall known diversity of AAP in pure culture collections. Strain BL67 is of immediate interest, a relative of *Sandarakinorhabdus cyanobacteriorum* with novel LHII complex, the first AAP discovered with a peak at 855 nm absorbance and requires follow-up membrane extract analysis to confirm the LH complex composition. The *Rubellimicrobium rubrum* related AC101 strain is also compelling due to its lower 16S rRNA sequence similarity around 96.16% to a non-phototrophic genus and having unusual constricted single-cellular morphology. Likely both strains can be classified as new species and potentially genera. In addition, if DNA from all 20 species are sequenced, photosynthesis regulating genes could be compared, and this will provide new insights into AAP physiological capabilities and controls, as only very few studies have investigated their genetic arrangements (Zheng *et al.*, 2011). As we already collected absorbance spectra for all 20 species representatives, genome sequencing would open insights into what genetic varieties provide configurational changes and specific absorbance maxima. In addition, many physiological traits were determined in our work, including metal(loid) oxide reduction activities. These functions could also be linked to genes by analyzing annotations, or by future molecular genetics approaches that could identify key enzymes. Since we now possess many strains from a number of families, and they all had comparable activity towards metal(loid)s, each will likely have similar enzyme composition for these transformations.

Furthermore, in this work AAP were found to have additional metal(loid) activity through the production of not only siderophores, but metallophores as well (Chapter 5). Follow-up work can determine if AAP capable of sequestering multiple metals do so by producing a single specific secondary metabolite that acts on several elements, or multiple metallophores made for each individual cation. As some strains chelated all

10 metal(loid)s, a larger range of cations should be tested to determine the functional extent of the CAS method, and of the AAP capability/ tolerance. Additionally, the brown pigmented siderophore of *Chromocurvus halotolerans*, EG19^T would benefit from structural characterization, followed by a few other stronger metallophores produced by *Erythromonas ursincola*, KR99^T.

While deep violet coloured heterotrophic bacteria were discovered (Chapter 6), they did not contain photosynthetic pigments such as BChl a. Yet, the novelty of violet aerobic bacteria is staggering, but little is still known about why these bacteria synthesize violacein, especially in cold environments. Later investigation into this pigmentation would be an asset, as the absorbance of light is typically connected to functionality. Furthermore, our study was the first to enumerate violacein producers in the natural environment and additional analysis is required to understand how prevalent they are geographically, particularly in other cold habitats.

Overall, our study of microbial communities in Lake Winnipeg provided clear evidence of diverse yet stable population and its dynamics that drastically changed seasonally, returning to a similar structure each year. As the first analysis of bacteria in this habitat, we provided ecological insights into the fluctuations of individual phototrophic populations, and discovered novel AAP strains and associated physiology, likely leading towards new species descriptions. The linking of multiple metal transformations and their direct association to available daylight strongly supported a specific niche for these phototrophs. Since many new and interesting bacteria were found in the 11th largest freshwater lake, later work will not only show how important our results are, but will also likely reveal Lake Winnipeg to have more hidden surprises for future microbiologists to discover.

7.3. Bibliography

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