MOLECULAR CHARACTERIZATION OF MICROBIAL COMMUNITIES IN THE SUNDAYS AND SWARTKOPS ESTUARIES IMPACTED BY ANTHROPOGENIC ACTIVITIES

A thesis submitted in fulfilment of the requirements for the degree of

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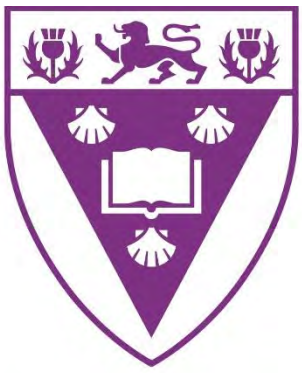

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Where leaders learn

By

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List of Abbreviations

ANOSIM – Analysis of similarities

BLAST - Basic Local Alignment Search Tool

bp – Base pairs

DNA – Deoxyribonucleic Acid

gDNA – Genomic Deoxyribonucleic Acid

NCBI – National Centre for Biotechnology information

NH_4 – Ammonium

NMDS – Non-metric multidimensional scaling

OTU – Operational taxonomic unit

PCoA – Principal co-ordinate analysis

PCR – Polymerase chain reaction

ppt – parts per thousand

rRNA – ribosomal Ribonucleic Acid

SRP - Soluble Reactive Phosphorus

TOxN – Total Oxidised Nitrogen

WWTP- Wastewater treatment plant

ABSTRACT

Anthropogenic activities are of concern in estuarine systems as they are the main source of water degradation. Water pollution in estuaries is indicated by eutrophication and the presence of pathogens and bacterial indicators which affect biodiversity and energy flow. This study focused on two geographically linked estuaries, namely the Sundays and Swartkops Estuaries. The Sundays Estuary is primarily impacted by agricultural activities in the river catchment with increased nutrients levels, particularly of total oxidised nitrogen (TOxN), likely derived from these farming activities. In contrast, the Swartkops Estuary, which is heavily influenced by urban/industrial activities, reflected increased levels of phosphates likely from wastewater and sewage water contamination from residential areas, leaking pipes, and poorly managed sewage treatment plants. The central objective of this study was to assess microbial population profiles and diversity impacted by agricultural activities in Sundays Estuary and industrial/urban-influenced Swartkops Estuary using 16S and 18S rRNA gene metabarcoding. A distinct difference in eukaryotic composition and diversity was evident between the two sampling exercises in 2018 and 2019 in Sundays Estuary. The eutrophication of both the Sundays and Swartkops estuaries was evident in the repeated occurrences of bloom events. In the Sundays Estuary, a bloom of *Heterosigma akashiwo* was observed in 2018 whilst *Cyclotella* dominated the estuary in 2019. The Swartkops Estuary exhibited seasonal variation in phytoplankton composition with *Bacillariophyceae* blooms in the upper reaches of the estuary in summer and increased prevalence of *Dinophyceae* in spring. Bacterial taxonomic variation was also noted between the two contrasting estuaries. Although members of the Proteobacteria dominated both estuaries, Gammaproteobacteria were in increased abundance in Sundays Estuary while members of Alphaproteobacteria were in high relative abundance in the marine dominated Swartkops Estuary. Members of the Bacteroidetes were the second most abundant bacterial phylum in both estuaries. Bacterial indicators of agricultural anthropogenic impacts identified in Sundays Estuary included members of *Sporichthyaceae, Erysipelotrichaceae*, *Nostocaceae*, and NS11-12_marine_group while some taxa such as the *Flavobacteriaceae*, *Cryomorphaceae*, and *Halieaceae* reflected their capability in degrading the phytoplankton bloom biomass present in the estuary. The urban impacts on the Swartkops Estuary was reflected by the contamination of the estuary with potential pathogens including *Aeromonas caviae*, *Vibrio fluvialis*, *Mycobacterium intracellulare*, *Vibrio cholerae*, and

Bacillus cereus. Bacterial community profiles of the major water inflow points into the Swartkops Estuary included members of the *Burkholderiaceae, Rhodocyclaceae, Aeromonadaceae*, and *Arcobacteriaceae* which are typically indicative of raw sewage contamination. The Motherwell canal, which runs through informal settlements, was the most polluted input source with high levels of anthropogenic nutrients and pathogenic bacteria. The Chatty river, which also runs through townships, recorded increased nutrient concentrations and low bacterial richness and diversity which was likely due to an *Arthrospira* bloom at the time of sampling. The overall results of this study identified sources of pollution in Sundays and Swartkops Estuaries and highlighted the impacts of anthropogenic inputs on microbial population profiles and diversity.

CHAPTER 1: LITERATURE REVIEW

Estuaries represent an interface between freshwater river systems and the marine environment (Adams *et al*., 2016). When marine water is pushed by tidal events towards the river, freshwater rises above the marine water, forming zones of salt stress of different pelagic subsystems, which makes estuaries the most complex and variable ecosystems on earth (Dame, 2018). The mixing of marine water and freshwater depends on tidal events and catchment rainfall (Reichelt-Brushett *et al*., 2017), and mixing zones vary because of uneven bottom profiles caused by erosion during storms. Some estuaries are permanently open to the ocean with good flushing systems while some are temporarily closed and open during floods resulting in long residence times (Perissinotto *et al.,* 2010). Estuaries are productive environmental ecosystems (Costanza *et al*., 1997) with diverse communities of prokaryotes and eukaryotes that feed, grow and replicate in the water (Dame, 2018). The typically shallow depth in estuaries makes it easier for benthic and pelagic organisms to mix in the water column and to form complex food webs. The ecological interaction in the estuaries which allows for energy flow (Kennish, 2014) has made it difficult to understand the effects of environmental conditions on ecosystem processes involving microbes and microalgae (James *et al.,* 2003). Estuaries are unique ecosystems influenced by physico-chemical conditions, tidal characteristics, sedimentation, hydrography, and geomorphology (Kennish, 2002), therefore, each estuary has a unique group of plants and animals, including many fish species that enter estuaries as migrants (Elliot *et al.*, 2007).

Estuarine systems maintain healthy conditions by filtering out toxic pollutants and trapping nutrients from the terrestrial environment before flowing into the oceans, hence provide cleaner waters for marine life (Dame, 2018; Adams, 2014). They are used for recreational and economic purposes as tourist attraction sites. Fishing is one of the common activities practiced in estuaries (Pita *et al*., 2017), and has resulted in high human population growth in the catchment (Kennish, 2002). High human population densities contribute to pollution in the estuarine systems

(Freeman *et al.,* 2019), which can be reflected by the microbial community composition associated with activities taking place in the catchment.

1.1 Environmental threats on estuaries

Anthropogenic activities emanating from rapid population growth include urbanization, uncontrolled industrial infrastructure, and agricultural activities, all of which contribute greatly to estuarine water deterioration (Kennish, 2002; Scharler *&* Baird*,* 2005a; Gyedu-Ababio, 2011; Malham *et al.,* 2014; Matcher *et al*., 2018; Adams *et al*., 2019). Although natural forces also play a role in changing the water quality in estuaries (Chapman, 2007), anthropogenic activities remain the greatest contributor to estuarine pollution (Kennish, 2002). Industrial effluents and sewage contamination have been identified as the main sources of pollution in most estuaries situated in urban areas (Ribeiro & Kjerfve, 2002; Taljaard *et al.,* 2017). Malfunction of sewage treatment plants results in the inflow of human pathogens into the estuaries which causes loss of ecological integrity. Estuaries are also polluted by small-scale anthropogenic inputs, including surface run-off, which may contain animal and human excretes (Fisher *et al*., 2015) as well as floatable debris and litter (Kennish, 2002). Litter and debris diminish the aesthetic value of estuaries and are unsafe to aquatic animals. Estuarine ecosystems are often impacted by the inflow of pathogens, chemical toxins, and the overloading of nutrients and organic matter from urbanized/industrialised and agricultural activities in the catchment area. Amongst these pollutants, organic compounds remain the greatest threat in heavily industrialized areas (Kennish, 2002).

The biggest threat in estuarine systems in South Africa is massive water abstraction which reduces freshwater input (Scharler & Baird, 2003). High human population densities along estuaries has resulted in overexploitation of resources, loss of estuarine natural state, change in species composition and distribution patterns, decrease in species diversity, and the inability of the estuary to recover from alteration (Lotze *et al.,* 2006; Neto *et al*., 2010; Dafforn *et al.,* 2012;

Stuart-Smith *et al*., 2015; Meziti *et al*., 2016; Adams *et al.*, 2020). Moreover, deterioration of estuaries can change estuaries from being highly productive to low productivity, with low richness and diversity, and change the filtering capacity of the system (Lotze *et al.,* 2006). The weak flushing estuarine systems are more likely to be impacted while well-flushed estuaries are healthier, however, this means that the pollutants have been washed out to sea which is not a good thing either. The modification of estuarine habitats disturbs the ecological functioning of the estuarine ecosystem, and it is a threat to the health of all living organisms and people living in the vicinity. An example is the filter-feeding shellfish that accumulate pathogens from contaminated estuarine waters and become a health risk to consumers (Malham *et al*., 2014).

1.2 Industrial and Urban Pollutants

Industries produce large amounts of toxic and pharmaceutical chemicals (Birch *et al.,* 2015) that may be discharged directly into the estuaries or enter estuaries through stormwater inflow. Other chemical pollutants from industries include aerosols and fossil fuels which cause air pollution and subsequently acid rain that can affect the physico-chemical status of water bodies. Some contaminants from industries include heavy metals which were found in juvenile stages of popular angling fish from estuaries (Nel *et al.,* 2015) and can influence the microbial composition in estuaries (Sheeba *et al.,* 2017). Urbanization contributes to estuarine pollution through deposition of utilized chemicals such as insecticides, herbicides, and pesticides that enter estuaries from lawns through surface run-off and faulty wastewater treatment plants which increase metal pollution in estuaries (Birch & Rochford, 2010) of which some are carcinogenic and may be a threat in higher trophic levels. Drug-resistant pathogens may accumulate in animal tissues and transfer to higher trophic levels (humans) through swimming or fish consumption (Ramirez *et al.,* 2009). Effects of industrialized and urbanized activities also include sediment deposition and nutrient loading which leads to eutrophication and harmful algal blooms (Dauer *et al*., 2000; Lemley *et al*., 2018b). Common anthropogenic sources of nutrients include sewage discharges and detergents in wastewater (Statham, 2012). Chemicals change the physico-chemical characteristics of water (Rajaram *et al.,* 2005; Gaw *et al.,* 2014) and the color

of water resulting in loss of aesthetic value, as well as abundance and diversity of biological communities in the estuarine ecosystem. As biological populations increase due to high nutrient levels, competition for oxygen by aquatic organisms increases, and fauna tend to consume more oxygen during effluent stress (Soundarapandian *et al*., 2009).

Many microbial species are present in estuarine ecosystems as a result of pollution and contamination of estuarine systems. The ability of microbes to potentially degrade pollutants is a reflection of their diverse metabolic activities which help in breaking down complex chemicals and organic matter (Jia *et al*., 2019). For example, microorganisms such as *Arthrobacter, Bacillus, Flavobacteria, Micrococcus, Mycobacterium,* and *Pseudomonas* are capable of oxidizing and degrading both aliphatic and aromatic hydrocarbons and contribute to their elimination (Das & Chandran, 2011), and therefore can be associated with industrial/urbanised pollution. Other bacterial communities that can be associated with urbanized pollution include *Arcobacter* and *Acinetobacter* which are commonly identified in sewage water (Fisher, 2015).

1.2.1 Industrially Impacted Estuary – Swartkops

Swartkops is one of the few urbanized medium-large estuaries in the warm temperate climate of South Africa and forms an integral part of Gqeberha (formerly known as Port Elizabeth) in the Eastern Cape (Nel *et al.*, 2015). The Swartkops Estuary, which is 16 km long, flows through a highly urbanized and industrialized area. It discharges into Algoa Bay ~11 km north of Gqeberha (Figure 1.1) (Emmerson, 1985), and has a catchment area of approximately 1360 km^2 (Baird *et al*., 1986). Swartkops river has one impoundment, the Groendal dam, that supplies the industrial area of Uitenhage with freshwater (Emmerson, 1985). Swartkops has a relatively pristine catchment at the upper reaches and has the third-largest salt marsh nationwide (Colloty *et al*., 2000), however, the middle and lower reaches of the Swarkops river and estuary have been severely impacted, particularly in recent years. It is in the 11^{th} position out of more than 280 estuaries in South Africa in terms of its biodiversity, habitat, and size (Turpie *et al*., 2002) and is

ranked 3rd with regards to national botanical importance (Colloty *et al*., 2000). This makes the increased anthropogenic pollution and degradation of the estuary over the last decade particularly distressing.

The Swartkops Estuary is an important recreational and ecological asset to people living in the surrounding areas of Gqeberha, even though it is impacted by industrial activities (Nel *et al.,* 2015). Industrial activities along the Swartkops Estuary include sewage treatment plants, salt works, clay mining, wool washers, and tanneries (Binning & Baird, 2001). The area bordering the estuary also includes the Algorax carbon factory, Swartkops power station, and the South African Transport Services yards (Hilmer & Bate, 1987). Residential areas closer to Swartkops Estuary, such as Swartkops village, Redhouse and Amsterdamhoek also contribute to pollution through deposition of litter and discharge of wastewater. Although some places like Motherwell and Kwazakele are a distance away from Swartkops Estuary, they contribute to pollution through water inflow via water channels which directly connect these areas to the Swatkops Estuary (Adams *et al.,* 2019). The lower to middle reaches of the estuary are partially fed by the Motherwell and Markman canals and Chatty river which are the largest influents on the estuary. Moreover, Tippers creek which flows through the urbanized residential areas discharges into the lower reaches of the estuary (Nel, 2015). The constantly flowing Chatty river flows through informal settlements (Scharler & Baird, 2003), and discharges pollutants from stormwater run-off, litter, and untreated sewage water from the townships (Adams *et al.,* 2019) into the heavily urbanised lower reaches. The Motherwell and Markman canals discharge pollutants and contaminants from the leaking raw sewage pumps and litter into Swartkops Estuary from the industrial, township, and residential areas (Adams *et al.,* 2019). Some treatment waterworks near the Swartkops Estuary are unable to adequately process the large volumes of sewage entering into the treatment plants and the sewage ends up flowing into the estuary. The Swartkops river which discharges water into the upper estuary is affected by the effluents from the poorly maintained wastewater treatment plants, as well as the Uitenhage residential area. Industrialisation and urbanisation increased inorganic nutrient load and heavy metal

accumulation within the sediments in the Swartkops Estuary (Binning & Baird, 2001). A study carried out by Adams *et al*. (2019) showed high nutrient concentrations in Swartkops Estuary which resulted in high phytoplankton biomass. Eutrophication in Swartkops resulted in the accumulation of the most problematic weed in South Africa, water hyacinth, which increases evaporation rate and obstructs waterways in the estuary (Chamier *et al*., 2012; Adams *et al.,* 2020). The estuary has also been reported to have high *Escherichia coli* and *Enterococci* levels which makes it hazardous for recreational purposes (Adams *et al*., 2019). These anthropogenic inputs have modified the estuary to category D of the Present Ecological State (PES) (Adams *et al*., 2019).

Figure 1.1: Overview of a map of Swartkops Estuary and part of the catchment contributing to water pollution. The map was generated using Google Earth Pro V. 7.3.3.7786

1.3 Agricultural Pollutants

Agricultural return flow in water sources has been reported to be the main cause of pollution followed by urban runoff, and wastewater effluents (Carpenter, 1998; Scharler & Baird, 2000; Taljaard *et al*., 2018; Adams *et al*., 2019). Excessive use of fertilizers from agricultural activities causes eutrophication, high turbidity, and conductivity and subsequently changes the biodiversity of aquatic communities in estuaries (Chen *et al*., 2018). Eutrophication obstructs sunlight and oxygen from reaching benthic aquatic organisms and causes hypoxia which slows down respiration, resulting in phytoplankton decay and increased organic matter. Some phytoplankton such as the blue-green, red, and brown algae release toxins that can change the biodiversity of biological inhabitants (Howarth *et al*., 2000; Bukaveckas *et al.,* 2018).

Agricultural pollutants enter estuaries through land and stormwater runoff from agricultural farms which may carry fertilizers from animal manure and synthetic fertilizers containing phosphorus and nitrogen in different forms i.e. nitrate, ammonium salts, and organic nitrogen compounds (Kennish, 2002). Nitrogen and phosphorus are in increased amounts in estuaries due to human activities (Statham, 2012) and have been indicated as the major sources of pollution (Howart *et al*., 2002). Nitrogen is changed by bacterial activities to ammonium compounds and oxidized to nitrites and finally to nitrates (Sliekers *et al*., 2005). Nitrites from agricultural fertilizers cause brown blood disease in fish resulting in subsequent negative impacts on food webs in estuaries, and humans as the last consumers in the food web (Esmail *et al.,* 2015). High amounts of ammonia in estuaries may be due to the death and decomposition of phytoplankton and excretion of ammonia from planktonic organisms (Soundarapandian *et al*., 2009) and ammonium in estuarine systems play a role in the cycling of organic matter (Statham, 2012). Nitrogen is usually reported as nitrate because of its stable condition under oxic conditions and it is mostly associated with eutrophication (Statham, 2012). The type of energy cycling in estuaries is determined by the amount of organic and inorganic nutrients, and the estuaries are either in autotrophic or heterotrophic conditions (Hopkinson & Smith, 2007). Heterotrophic conditions occur when bacterial respiration exceeds that of phytoplankton (Heip *et al.,* 1995)

while autotrophic conditions occur when there is high primary productivity. The amount of nutrients in estuaries determines the type of inhabitants. Nutrients in decreased amounts attract small-sized prokaryotic heterotrophs because of their high surface to volume ratio, while high nutrient content attracts larger cells such as osmotrophs, Cyanobacteria, and eukaryotic photoautotrophs which have high internal storage capabilities (Cotner & Biddanda, 2002).

Farms discharge chemicals, organic matter, and drug residues into sediments and estuarine water bodies. Agricultural runoff or drainage of chemicals affects microbial community composition (Montuelle *et al.,* 2010) by selecting bacterial communities that can survive the chemicals and nutrients pollutants. Some microbial species, especially those of the genus *Pseudomonas,* are capable of breaking down pesticides in aquatic systems by using pesticides as a source of carbon, nitrogen, and energy (Barra *et al*., 2013; Kahlon, 2016). Many of the organochlorines and organophosphates used in agricultural fields can exist in the environment for a long time and degrade slowly, with some absorbed by organisms (Reichelt-Brushett *et al*., 2017; Sruthi *et al.*, 2018).

1.3.1 Agriculturally impacted estuary – Sundays Estuary

Sundays Estuary (33°43’S, 25°25’E) (Figure 1.2) is situated in the Eastern Cape on the southeast coast of South Africa, approximately 40 km northeast of Gqeberha (formerly known as Port Elizabeth) (Lee & Du Preez, 2015). The estuary is ~24 km long, permanently open with continuous freshwater inflow, and discharges into Algoa Bay (MacKay & Schumann, 1990). The catchment area of the estuary is approximately 22 000 km^2 and comprises predominantly of the arid Karoo, surrounded by coastal dune fields and steep banks with no salt marsh (Beckley, 1984). The Sundays Estuary has a constant freshwater inflow from its catchment area despite two major impoundments, namely the Van Ryneveld and the Darlington dams (Scharler *et al.,* 1997). Sundays Estuary receives freshwater from the Sundays river which runs through part of the Addo elephant park (Kotsedi *et al.,* 2012). Freshwater inflow in the Sundays Estuary is supplemented

by an inter-basin transfer scheme from the Gariep river system (Emmerson, 1989) and one of the largest rivers in South Africa, the Orange river (Lee & Du Preez, 2015). As with the Swartkops, the estuary is used for recreational purposes such as bird watching, boating, and sandboarding. Sundays Estuary is ranked 39th in terms of its biodiversity conservation importance (Turpie *et al*., 2002).

Anthropogenic input into the Sundays Estuary is mostly from farming activities particularly at the upper reaches where there are citrus farms and excessive use of fertilizers. Sundays Estuary has no tributaries (Scharler & Baird, 2005b) but rain and winds carry large amounts of nitrogen and phosphorus from the agricultural fields into the estuary (Khatri & Tyagi, 2015). High levels of nutrients and phytoplankton production have been recorded in Sundays Estuary (Lemley *et al*., 2017). Lemley *et al*. (2017*,* 2018b) reported Sundays Estuary to be permanently eutrophic with high chl-*a* content, due to high phytoplankton biomass with aquatic invasive plants upstream which leads to bottom water hypoxia and subsequent massive killing of fish. Harmful algal blooms previously identified in Sundays Estuary include that of the *Heterosigma akashiwo* and *Heterocapsa rotundata* and submerged macrophytes, *Stuckenia pectinate,* were found at the upper reaches of the estuary (Adams *et al*., 2020).

A

B

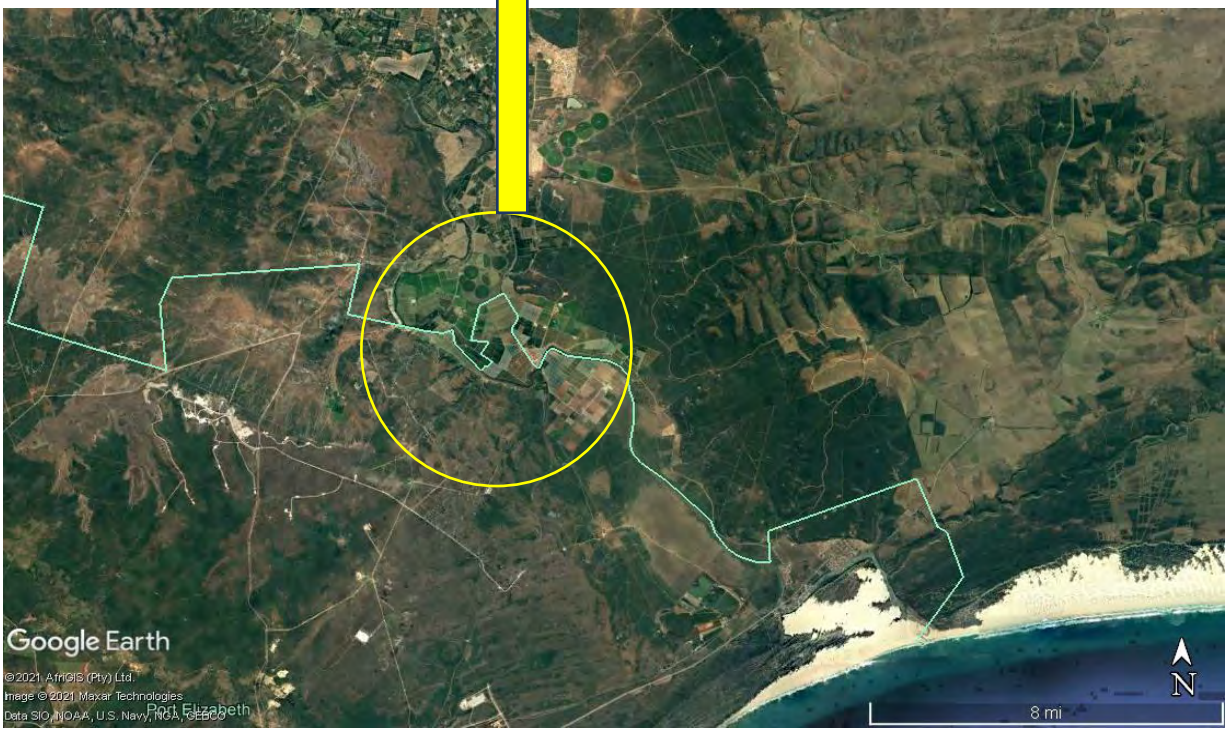

Figure 1.2: Overview of a map of Sundays Estuary (A) and an enlarged picture of an example of the agricultural farms contributing to pollution in the upper catchment (B). Blue line: path of the river. Map was generated using Google Earth Pro V. 7.3.3.7786

1.4 Phytoplankton in estuaries

Estuaries are a habitat for different types of phytoplankton that form the base of energy flow and sustain other biota such as microbiota, fish, and benthic organisms. Phytoplankton use inorganic nutrients for production (Hopkinson & Smith, 2007), therefore, their prolific growth indicates the presence of excess nutrients (Hall *et al*., 2013) and can be used as indicators of the health status of estuarine systems. Nutrients that stimulate phytoplankton productivity in estuaries mostly contain nitrogen and phosphorus from agricultural sources and municipal wastewater treatment plants which enter the estuary through freshwater inputs (Kennish, 2002; Dalu *et al*., 2018). In addition to nutrients, the growth of phytoplankton is also limited by light, osmotic stress, grazing pressure, and residence time of the cells within the estuarine system (Statham, 2012). Phytoplankton differ in nutritional requirements, cell size, motility, and biochemical compositions (Litchman & Klausmeier, 2008) and their growth occurs mostly during intermediate flows, as heavy flows can flush them out from the estuary (Peierls *et al*., 2012). Different phytoplankton have different salinity growth requirements (Kouhanestani *et al*., 2019) and their biomass is stimulated by temperature changes and winds (Shikata *et al*., 2008a).

Pollution severely affects phytoplankton accumulation in many estuaries as it has results in the loss of some phytoplankton species in estuaries (Fowles *et al.,* 2018). Unlike in oceans, estuaries are commonly dominated by phytoplankton with large cells due to high nutrient inputs (Cloern, 2018). During phytoplankton blooms, oxygen content decreases, which supports Howell & Simpson, (1994) findings that showed a correlation between low dissolved oxygen and low phytoplankton species diversity. Overgrowth of phytoplankton caused by high nutrient levels has negative impacts on other aquatic biota as a result of obstruction of light and oxygen penetration to benthic organisms. Toxin-producing blooms may cause death of non-toxic phytoplankton and impacts on phytoplankton diversity (Kong *et al*., 2018).

Phytoplankton growth contributes to the production of organic matter and carbon fixation in estuaries (Lemley *et al*., 2016). Phytoplankton exudates can influence the composition, abundance, and diversity of bacterial populations (Bent & Goulder, 1981; Gasol & Duarte, 2000). Different phytoplankton play different roles in estuaries; Diatoms are important in silicon cycling (Yool & Tyrrell, 2003), blue-green algae play roles in carbon, oxygen, nitrogen, and phosphorus cycling, and some such as diatoms, Dinoflagellates, and Cryptophytes are rich in fatty acids hence nutritious to consumers (Peltomaa *et al*., 2019). However, the over-abundance of these phytoplankton communities can be harmful and result in water degradation (Carstensen *et al*., 2015). Diatoms grow rapidly under high nitrate uptake and can adjust to different light intensities in the water column (Lomas & Gilbert, 2000). Previous studies showed that diatoms in estuaries mostly dominate phytoplankton blooms followed by Dinoflagellates (Sarthou *et al*., 2005; Carstensen *et al*., 2007; 2015). Even though phytoplankton dynamics remain poorly understood due to the interaction of biological and chemical processes in the estuarine system (Cloern, 2018), they play important ecological and biogeochemical roles in aquatic systems (Carstensen *et al*., 2015).

1.5 Role of bacteria in estuaries

Bacteria are ubiquitous and abundant as well as phylogenetically and metabolically diverse microbes that form a significant part of the ecosystem. They form the major component of aquatic environments as important drivers of energy flow and play a critical role in regulating biogeochemical nutrient cycling needed for the growth of plants and animals such as in nitrogen, sulfur, carbon, and phosphorus (Colwell, 1978; Middelburg & Nieuwenhuize, 2000; Cotner & Biddanda, 2002; DeLong & Karl, 2005; Cole *et al*., 2007; Rastogi *et al*., 2011). Bacteria also degrade pollutants in estuaries (Rasul & Chapalamadugu, 1991) and contribute to the oxygen content (Biddanda *et al*., 2001). Some bacteria are algicidal (Manage *et al*., 2001) and help in balancing the estuarine ecosystem.

When bacteria enter estuaries, they either remain in the water column as free-living cells or, more commonly, form particle-associated communities (Goulder, 1977). Alternatively, bacteria entering the estuary may settle to the bottom of the estuary which, in the case of bacterial pollution from sewage-derived sources, generates concerns for the recontamination of the estuary when the sediment is agitated and re-suspended in the water column (Jeng *et al.,* 2005). Both particle-attached and planktonic bacteria degrade detrital organic matter made available by phytoplankton and macroalgae which are consumed by detritivorous copepods (Bent & Goulder, 1981; Ducklow *et al.,* 1993; Simenstad *et al*., 1994; Cole *et al*., 2007; Rastogi *et al*., 2011), rotifers, and protozoa (Crump & Baross, 1996).

Research studies have used bacteria as biological indicators of pollution due to their association and capability to degrade biological and chemical pollutants (Lemke *et al.,* 1997). Their metabolites increase mineral dissolution in surrounding areas and reduce toxicity through active uptake and passive absorption (Gadd & Griffiths, 1977). Even though a decrease in bacterial diversity is used to represent a decline in ecosystem health (Allison & Martiny, 2008), the response of prokaryotes to contaminants is complicated (Sun *et al*., 2012). Some bacteria are sensitive to contaminants while some grow in the presence of toxins (Gadd & Griffiths, 1977).

1.6 Factors potentially influencing bacterial composition, diversity, and distribution patterns in estuaries

Dynamic changes in estuarine ecosystems provide bacteria with new growth opportunities. A previous study by Zhang *et al*. (2020a) indicated that bacterial structure is closely related to water quality. However, there are no clear conclusions on factors influencing bacterial composition, diversity, and distribution (Newton & McLellan, 2015), with very little known about their growth rates and response to alteration of environmental conditions such as temperature, residence time, nutrient concentrations, conductivity, total dissolved solutes, dissolved oxygen, and salinity (Colwell, 1978; Bent & Goulder, 1981; Allgaier & Grossart, 2006; Nocker *et al.,* 2007; Newton *et*

al., 2011; Mai *et al*., 2020). While the rate of mixing of marine and freshwater has been shown to have a great impact on bacterial distribution patterns (Wright & Coffin, 1983), Crump *et al*. (2004) stated that chemical and biological conditions that drive microbial shifts are unknown, and the results of the study showed that bacterial production was constant along the salinity gradient, and high bacterial production was associated with seasonal phytoplankton blooms. Bouvier & Del Giorgio (2002) indicated that the distribution of bacterial communities in estuaries is determined by salinity gradient as salt-loving bacteria, Alphaproteobacteria, were found in water with a high salinity gradient, while Betaproteobacteria were found in the freshwater zones. Shifts in microbial communities are commonly influenced by environmental variations and anthropogenic inputs such as increased nutrient concentrations and chemical inputs, hydrocarbons, and industrial effluents (Jeffries *et al*., 2016; Crump *et al*., 1999). Only stress-tolerant organisms which can withstand sharp physico-chemical gradients can be found in estuarine ecosystems, therefore, estuaries are typically characterized with a relatively low species richness as compared to freshwater and full salinity conditions (Nebra *et al*., 2016).

Nutrients from anthropogenic activities and salinity have been highlighted as the most common factors affecting microbial diversity in estuaries (Dame, 2018; Guo *et al.,* 2017). Bacterial populations in estuarine conditions have been referred to as r-strategists i.e. survive in nutrient rich environments (Poindexture, 1981). Microbial abundance and activity are usually higher in eutrophic conditions (Cotner & Biddanda, 2002) but can be reduced by grazing (Sanders *et al*., 1992) and viral mortality (Weinbauer & Peduzzi, 1995). Eutrophication is one of the factors that influence the composition of microbial communities as some algal species release toxic metabolites that some microbes cannot withstand in estuaries (Liu *et al*., 2013), however, high proliferation of bacteria can also build up toxic material and by-products of decomposition (Downey, 1976). Bacteria respond to eutrophication by shifting substrate uptake capabilities, increasing cell size and the proportion of actively respiring cells (Yager *et al.,* 2001), and altering bacterial surface area (Stoderegger & Herndl, 2005).

1.7 DNA Metabarcoding

The identification of microbial communities has been a hurdle in research for the past years. DNA metabarcoding has now emerged as a powerful tool that is frequently used for characterization of microbial communities (Francioli *et al.,* 2021). The reason for developing new technology in microbial identification is due to the limitations of the conventional methods which include the need for taxonomic expertise, time consumption, cost ineffectiveness, and misrepresentation of the microbial composition (Thomsen & Willerslev, 2015). DNA metabarcoding is currently the preferred method in bacterial community profiling and uses short genetic target regions, such as the regions of the 16S rRNA, to identify millions of diverse bacteria within microbial communities (Bush *et al*., 2020; Francioli *et al.,* 2021). It is the most reliable, accurate, and cost-effective molecular method of microbial identification (Liu *et al*., 2020) and is useful in the rapid detection of environmental changes such as in the estuarine systems, detection of broader taxa per sample, and is efficient at a finer taxonomic resolution (Bush *et al*., 2020). DNA metabarcoding consists of laboratory steps which include the extraction of genomic DNA, amplification of the targeted barcode gene, genomic sequencing of the amplicon, and bioinformatics analysis and computational statistics (Yu *et al.,* 2012; Francioli *et al.,* 2021).

Although the conventional methods of DNA extraction which involve the use of chemicals such as the phenol-chloroform based extraction methods are used for research (Griffiths *et al.,* 2000), commercial DNA extraction kits are mostly used particularly in metabarcoding (Liu *et al*., 2020). Successful DNA extraction occurs when cells are completely disrupted, protein and nucleoproteins denatured, and PCR inhibitors removed from the genomic DNA (Francioli *et al.,* 2021). DNA metabarcoding is based on high throughput sequencing of a specific DNA marker (Liu *et al*., 2020) amplified by PCR (Compson *et al.,* 2020). Different groups of organisms have different taxonomic informative gene targets/markers that have been tested. It is very important to select a suitable barcode locus and PCR primer set before carrying out the metabarcoding analysis as the correct and adequately informative detection and identification of target populations rely on this (reviewed by Piper *et al.,* 2019). Where the target population is not well

known, universal primers may be used to encompass a wide range of species (Heinze, 2007). In the case of bacteria, the 16S rRNA gene has been identified as the gold standard for bacterial analysis (Machida *et al.,* 2012; Pavan-Kumar *et al.,* 2015) while the 18S rRNA have been used for microbial eukaryotes (Hadziavdic *et al*., 2014). Whilst a powerful tool is used, it must be kept in mind that metabarcoding is dependent on PCR and the process of amplifying genomic DNA has limitations such as errors including substitutions and deletions/insertions (Taberlet *et al*., 2012). Suitable quality filtering of the resultant sequence data is critical in order to mitigate these potential errors. Raw sequence data of metabarcode libraries are typically generated on either the Illumina or Ion Torrent sequencing platforms. The resulting raw sequences are then processed through bioinformatics which involves quality filtering (Compson *et al.,* 2020) to limit or remove false positives (Thomsen & Willerslev, 2015). Identification of DNA sequences is done by comparative sequence alignment against DNA reference databases (Thomsen & Willerslev, 2015). Some of the most widely used reference databases used to identify DNA sequences in metabarcoding are GenBank and Barcode of Life Data Systems (BOLD) (Compson *et al.,* 2020). Not all organisms have been genetically characterized to date and will therefore not be present in the reference database. This is particularly true of bacteria which are extremely diverse and many of which are novel. Whilst sequence data generated from organisms not represented in the reference databases means that all the taxa present in the study sample cannot be elucidated from the resulting sequence datasets, taxonomy-free analyses can still be conducted to investigate variation in samples.

1.8 Research rationale and aims

The health of estuaries is critical to the survival of many fish, plant and animal species and for sustainable biodiversity. The world is experiencing a high frequency of anthropogenic modifications in estuarine environments and it is of fundamental importance to identify sensitive indicators of environmental stress in order to manage estuaries and prevent the extinction of species dependent on estuaries (Sun *et al*., 2012).

Whilst there are many ways of assessing pollution in estuarine systems, previous research studies on the assessment of anthropogenic inputs on Sundays and Swartkops Estuaries were based on algal blooms, phytoplankton communities, and fish assemblages which excludes bacterial communities. Complementary information which includes molecular characterization of bacterial and eukaryotes populations is important to understand the ecological perturbations in the estuaries. Bacteria and phytoplankton play critical roles in ecosystem functioning, but there is little knowledge on the impacts of pollutants on their composition and diversity in estuarine systems. Previous studies have indicated microbial communities as good indicators of stress due to their sensitivity to rapid changes in the environment (Paerl, 2006), therefore it is ideal to assess pollution in estuaries by examining bacterial and micro-eukaryotes assemblages in estuaries (Bouvier & Del Giorgio, 2002). Most of the work done to date on the Sundays and Swartkops Estuaries involves physical/microscopy identification. Whilst some limited research on bacterial populations was conducted in the Sundays Estuary (Matcher *et al*., 2018), no research on bacterial populations and diversity has been conducted in Swartkops Estuary.

The main aim of this research was to provide a fundamental understanding of the microbial communities present in Swartkops and Sundays Estuaries impacted by agricultural (Sundays Estuary) versus urban/industrial (Swartkops Estuary) activities. The study documented the response of microbial populations to environmental challenges and anthropogenic inputs by characterising shifts in the microbial population structure which would result in consequent changes in metabolic potential.

The objectives included:

- Comparative analysis of the agricultural influenced estuary by comprehensive mapping of the bacterial and micro-eukaryotic distribution and diversity profiles along the length of Sundays Estuary. (Chapter 3)
- Seasonal monitoring of the bacterial populations in the urban/industrial influenced Swartkops Estuary and identification of potential sources of pollution and their impacts in the estuary. (Chapter 4)

- Characterization of the bacterial composition within the water columns of water inflow channels into the Swartkops Estuary with a specific focus on harmful algal blooms, potential human pathogens and fecal indicator species. (Chapter 5)

CHAPTER 2: SITE DESCRIPTION AND RESEARCH METHODOLOGY

2.1 Site description

The Sundays and Swartkops Estuaries are geographically linked and situated in the temperate climate of the Eastern Cape, South Africa (Figure 2.1). The two estuaries both discharge into the Algoa Bay.

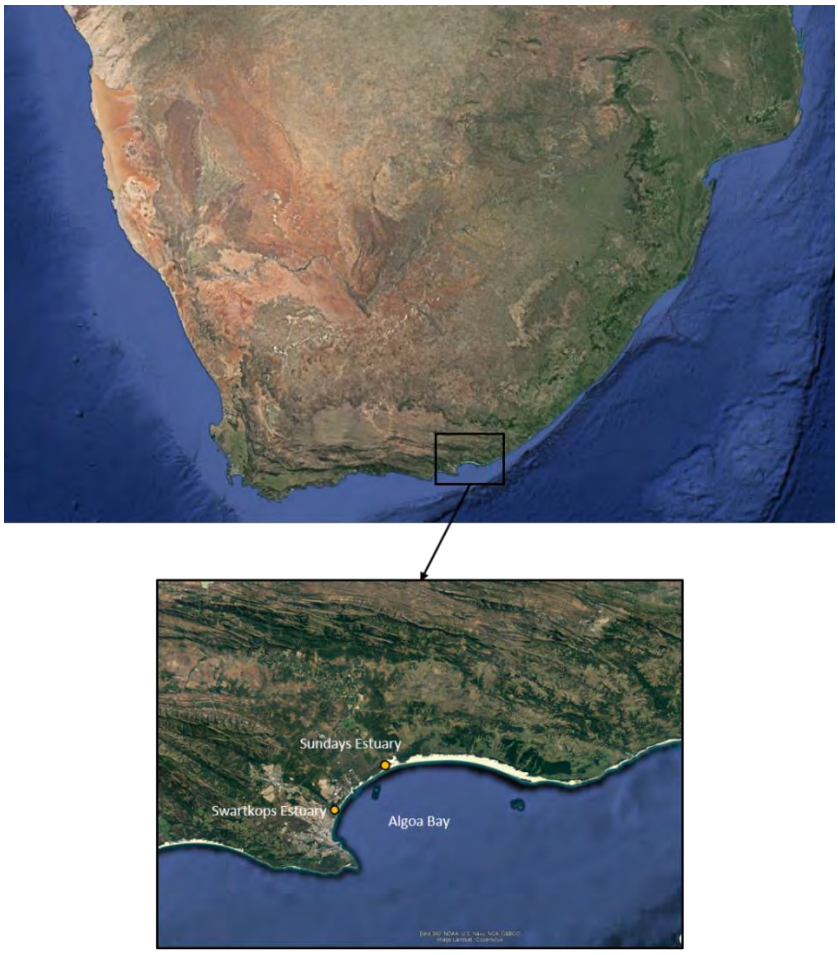

Figure 2.1: Geographical location of Sundays and Swartkops Estuaries

2.1.1 Sundays Estuary

Sundays Estuary (33°43'S, 25°25'E) is situated in the temperate region of the Eastern Cape, on the southeast coast of South Africa approximately 40 km northeast of Gqeberha (Lee & Du Preez, 2015). The estuary is ~24 km long, permanently open, with continuous freshwater inflow, and discharges into Algoa Bay (MacKay & Schumann, 1990). Sundays Estuary is impacted by farming

activities at the upper reaches (Figure 2.2) which makes the estuary an interesting area of research on the assessment of anthropogenic impacts on the biological structure of the system. The study area covered ~21 km of radius from the mouth to the upper reaches of the estuary (Table 2.1).

Figure 2.2: Aerial view of sampling sites along the length of Sundays Estuary (B) with an enlarged picture of agricultural farms (A) located in the catchment in the upper reaches. The map was generated using Google Earth Pro (v7.3.3.7786).

2.1.2 Swartkops Estuary

Swartkops Estuary is located ~11 km north of Gqeberha, and discharges into Algoa Bay (Emmerson, 1985). The Swartkops Estuary is ~16 km long and has a catchment area of

approximately 1360 km^2 (Baird *et al*., 1986) and is heavily urbanized/industrialised in the lower reaches with a relatively pristine catchment in the upper reaches (Colloty *et al*., 2000). The study sites covered ~16.3 km radius from the mouth of the estuary (Table 2.2). Swartkops Estuary receives water from the canals, rivers, and creek that run through urbanized/industrialised and residential areas. These include Tippers creek, Chatty river, Markman and Motherwell canals and Swartkops river (sampling points: Perseverance bridge, Nivens bridge, Bulmers drift, and Springfontein) (Figure 2.3).

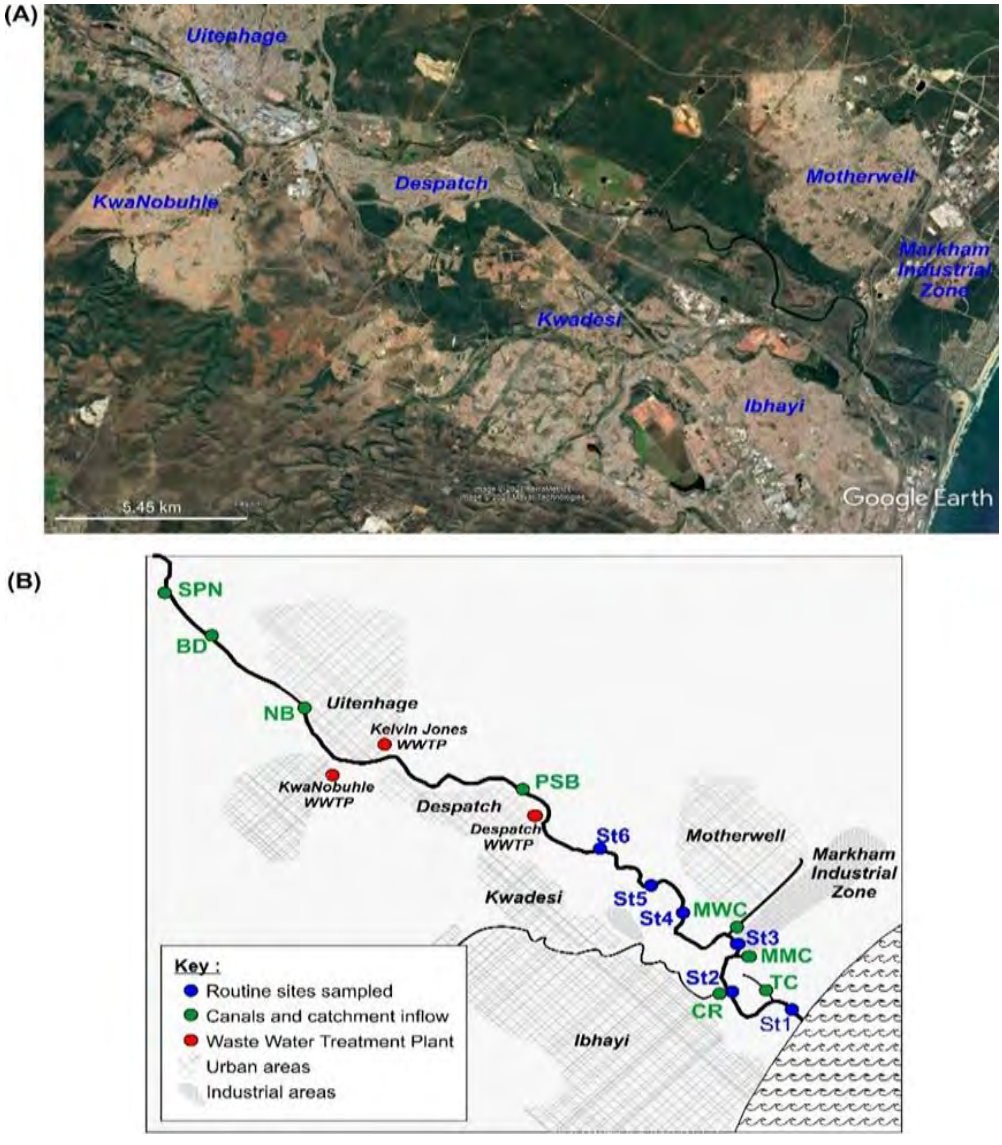

Figure 2.3: Aerial map (A) and geographical map (B) of sampling sites along the length of Swartkops Estuary and the rivers, canals, and creek flowing into the estuary. (TC: Tippers Creek, CR: Chatty River, MMC: Markman Canal, MWC: Motherwell Canal, PSB: Perseverance Bridge, NB: Nivens Bridge, BD: Bulmers Drift, SPN: Springfontein, St: Site)

The Chatty river flows through the informal densely populated settlements of Zwide, Bethelsdorp, Veeplaas, New Brighton, and Missionvale where the river is mostly contaminated by stormwater from residential areas (Scharler & Baird, 2005b; Adams *et al.*, 2019). Tippers creek runs through national roads, highways, and the urbanized Amsterdamhoek residential area. Both the Chatty river and Tippers creek discharge into the heavily urbanized lower reaches of the estuary. The Motherwell and Markman canals discharge stormwater into the middle reaches of the estuary (Scharler & Baird, 2003). The Markman canal is heavily influenced by industries and runs through a semi urbanised area (Adams *et al*., 2019) whilst the Motherwell canal flows through a low-income residential area (Motherwell). Before reaching the estuary, water from the Motherwell canal passes through an artificial wetland which was constructed to filter out contaminants (Adams *et al.,*2019). The upper reaches of the estuary receive water from the Swartkops river which had four sampling sites namely, Perseverance bridge, Nivens bridge, Bulmers drift, and Springfontein (Figure 2.3). Swartkops river receives stormwater from its tributaries such as the Elands river situated on the south side of the river, Kwa-Zunga situated on the north side of the river (Maclear, 1996), and the Kat canal which discharges some few meters upstream of the Nivens bridge sampling site. The Bulmers drift and Springfontein sampling sites are situated upstream in the Swartkops river where freshwater flows through into the estuary. The lower reaches of the Swartkops river is impacted by three wastewater treatment plants, Kelvin Jones, Despatch, and KwaNobuhle (Figure 2.2) which sometimes break down and discharge untreated wastewater into the river.

2.2 Sample collection

Sundays Estuary was sampled annually for two consecutive years during neap tides in the spring season (25th October 2018 and 15th November 2019). The water samples were collected from the surface, middle of the water column (~1 m depth), and near bottom of the water column from eight sampling sites (Table 2.1) between 08:50 hrs and 13:15 hrs. Samples were collected from the surface to the bottom of the water column and each sample was collected in single-use UV sterilsed bottles. The boat was switched off and water in the estuary was left to settle before sampling. The Sundays Estuary varied in depth from 1 – 2.5 m with a shallow depth (1 m) at site

2 at the time of sampling in 2018. A weighted pop bottle was used to collect replicate 500 mL of water samples from the middle of the water column (1 m) and near bottom of the water column, while bottles were dipped slightly in the upright position in the estuary to collect the surface waters. Sampled water was placed into the UV sterilised plastic bottles, and temporarily stored in cooler boxes until processing in the laboratory on the same day of collection. Sampling sites and locations (coordinates) are described in Table 2.1.

Table 2.1: Sampling sites along the length of Sundays Estuary

Site	Distance from the mouth (km)	Coordinates	Description
1	2.6	S 33° 42’25.3”; E 25° 50’ 23.5”	Marine impacted
2	4.5	S 33º 41’29.7”; E 25º 49’ 54.3”	Marine impacted
3	8.6	S 33º 42’42.4”; E 25º 47’ 49.5”	Agricultural impacted
4	10.5	S 33º 41’43.0”; E 25º 47’ 40.7”	Agricultural impacted
5	13.8	S 33º 40’49.9”; E 25º 46’ 10.3”	Agricultural impacted
6	17.6	S 33º 39’28.7”; E 25º 44’ 22.8”	Agricultural impacted
7	20.1	S 33º 38’10.6”; E 25º 44’ 17.5”	Agricultural impacted
8	21.6	S 33º 37’32.7”; E 25º 43’ 52.5”	Agricultural impacted

Swartkops Estuary was sampled seasonally during autumn (25th April), winter (25th July), spring (4th November) in the year 2019, and summer (4th February) in 2020. The water samples were collected similarly to what was described above, but the surface depth ranged between 0 – 0.2 m, middle (1 – 2.5 m), and the bottom of the water column ranged between 1.7 – 5.3 m. A total of 6 sites were identified as sample collection sites (Table 2.2). Site 6 could not be accessed by boat so only surface water was collected at this site. The water samples were collected between 10:26 – 15:00 hrs.

Table 2.2: Sampling sites along the length of Swartkops Estuary

Sites	Distance from the Mouth (km)	Coordinates	Description
1	0.4	33°51’45.27’’S; 25°37’40.54’’E	Marine impacted
2	4.3	33°51’23.60’’S; 25°35’51.01’’E	Industrial impacted
3	6.7	35°50’17.81’’S; 25°35’50.72’’E	Industrial impacted
4	10	33°50’9.79’’S; 25°34’16.50’’E	Industrial impacted
5	13.6	33°49’13.66’’S; 25°33’3.33’’E	Industrial impacted
6	16.3	33°48’39.63’’S; 25°31’49.73’’E	Industrial impacted

Samples were also collected from potential sources of pollution into the Swartkops Estuary (viz. Tippers creek, Chatty river, Markman canal, Motherwell canal, Perseverance bridge, Nivens bridge, Bulmers drift, and Springfontein) (Table 2.3). The sampling of the inflow channels was done prior to the water entering the estuary. Surface water samples were collected at each of these sites and sampling was done concurrently with sample collection from Swartkops Estuary during the autumn season (25th April 2019) between 08:30 – 13:40 hrs.

Table 2.3: Sampling sites of potential sources of pollution in Swartkops Estuary

Sampling site	Closest receiving site in the estuary	Coordinates
Tippers Creek (TC)	Site 1	33°51'14.30"S; 25°36'42.29"E
Chatty River (CR)	Site 2	33°51'33.78"S; 25°35'39.69"E
Markman Canal (MMC)	Site 2	33°50'35.90"S; 25°36'17.41"E
Motherwell Canal (MWC)	Site 3	33°50'9.41"S; 25°35'35.39"E
Perseverance Bridge (PSB)	Site 6	33°47'31.61"S; 25°29'23.77"E
Nivens Bridge (NB)	Site 6	33°46'18.91"S; 25°23'11.00"E
Bulmers Drift (BD)	Site 6	33°45'7.35"S; 25°20'34.85"E
Springfontein (SPN)	Site 6	33°44'10.32"S; 25°19'11.02"E

The physical parameters including salinity (ppt), pH, temperature (°C), and dissolved oxygen (mg/L) were measured with YSI ProDSS multiparameter *in situ*, from the same depths the samples were collected from, in the estuaries and the canals, rivers, and creek flowing into Swartkops Estuary.

2.3 Nutrients Analysis

Concentrations of soluble reactive phosphorus/phosphates, ammonium, and total oxidised nitrogen (nitrate and nitrite) were measured in samples collected from Swartkops Estuary and Sundays Estuary. Silicate concentrations were also measured for the Swartkops Estuary samples. The flasks used for the determination of soluble reactive phosphorus/phosphates were acid washed with nitric acid and dried overnight. The samples (filtrate from each depth, as stated under section 2.5) were frozen at -20^{o}C until analysed at which time they were thawed, and a SEAL AutoAnalyzer3 HR (SEAL Analytical, Inc) was used to determine nutrient concentrations (Murphy & Riley, 1962; Emmerson, 1985; Grasshoff *et al*., 1983).

2.4 Chl-a and Phytoplankton analysis

The water samples for phytoplankton biomass (cell count and chl-*a* concentrations) were collected from the water column and immediately gravity filtered onboard through the micro glass fibre filter papers (Munktell MGC, 1.2 µm pore-size, 47 mm diameter) using plastic filter cups and syringe. The filter cups were rinsed between collections from each sampling site. The micro-glass fibre filter papers were folded in tin foil and frozen until processing at the Ocean science laboratory at Nelson Mandela University.

The micro-glass fibre filter papers were thawed and chl-*a* extraction was performed by soaking the filters overnight at 4^{o}C in glass vials containing 10 mL of 90% ethanol. Phytoplankton biomass (chl-*a* concentration) was determined by spectrophotometry using a Thermo Scientific^{TM} GENESYS^{TM} 10S UV-Vis spectrophotometer. Samples for phytoplankton community composition were primarily preserved in 1 mL of 25% glutaraldehyde solution. They were then stained with Rose Bengal and settled for 24 hours in a 26.5 mm internal diameter Utermöhl settling chamber before identification. Phytoplankton cell counts were performed and identified at taxon level of class using Leisca DMIL phase-contrast inverted microscope with a magnification of 630X (Snow *et al*., 2000). Phytoplankton cell counts were done by Dr Lemley from the Department of Botany at Nelson Mandela University, Gqeberha, South Africa.

2.5 Microbial Community Sampling

Each 500 mL water sample was filtered through 0.22 µm pore-sized membrane filter papers (Supor®-200, PALL) in a filter unit with a single-use disposable MicroFunnel (PALL). The samples were homogenized before filtration and the volume of the water filtered was dependent on turbidity. The more turbid the water was, the lower the volume processed through the filters due to the clogging of membrane pores. In cases where a filter membrane clogged, new filter membranes were used for the remaining sample. The filter papers were folded using sterile pairs of tweezers and immersed in RNALater (Ambion) for preservation, in sterile 1.5 mL tubes. Samples were frozen at -20^{o}C until DNA extraction was conducted. Approximately 45 mL of the filtrate (i.e. water that had passed through the 0.22um filter membranes) from each depth was placed in 50 mL falcon tubes for nutrient analysis and frozen until processing at Ocean science campus, Nelson Mandela University. The glass sidearm flask that was used to collect the filtrate was thoroughly rinsed between samples of different depths/locations.

2.6 Genomic DNA Extraction

Genomic DNA (gDNA) was extracted using ZymoBiomics™ DNA MiniPrep extraction kit (D4300) as per the manufacturer's instructions with a few modifications as outlined below. The frozen samples were thawed, and half of the filter paper for each sample was chopped into tiny pieces with a sterile blade in a sterile petri dish and placed in a 1.5 mL eppendorf tube. In order to ensure that none of the microbial cells were lost as a result of dislodging from the filter membrane into the RNALater storage solution, 500 mL of RNALater in which each of the filter membranes were stored was added to sterile 1.5 mL eppendorf tubes and centrifuged at 10 000 rpm for a minute and the supernatant was discarded. The lysis buffer from the commercial kit (ZymoBiomics™ DNA MiniPrep extraction kit (D4300)) was used to resuspend the resultant pellet from the eppendorf tube and added to the chopped filter membrane. The remainder of the DNA extraction procedure was conducted as per the manufacturers specifications as outlined in the ZymoBiomics™ DNA MiniPrep user's manual. Approximately 75 µL of genomic DNA was eluted and the DNA concentration measured with a nanodrop spectrophotometer (Thermo Fisher Scientific™, ND-2000).

2.7 Amplicon library preparation and Illumina sequencing

The V4-V5 hypervariable region of the 16S ribosomal gene (16S rRNA) found in bacteria was amplified using the forward primer (515F 5'-GTGCCAGCMGCCGCGGTAA-3') and reverse primer 926R (3'-CCGYCAATTYMTTTRAGTTT-5') (Walters *et al*. 2015) with suitable MiSeq adaptor sequences added on to these target-specific primer sequences. A 40 µL PCR mix of each sample containing the above-mentioned primers, KAPA HiFiTaq Polymerase ready mix (Roche), PCR grade molecular water, 3µL of 50% Tween 20 (UniLAB®) and 18 – 95 ng/µL of genomic DNA template were subjected to cycling parameters of 98°C for 5 minutes (1 cycle); 98°C for 45 seconds, 44°C for 30 seconds, 72°C for 1 minute (5 cycles); 98°C for 45 seconds, 50°C for 30 seconds, 72°C for 1 minute (20 cycles); and a final extension of 72°C for 5 minutes. The samples were run against the negative controls to check for contamination during DNA extraction and amplification.

The targeted V9 hypervariable region of the 18S rRNA gene was amplified from the same eluted genomic DNA used for the 16S rRNA amplification on samples collected from Sundays Estuary. The PCR reaction mix was the same as the above with the exception of primers. The primers used for the 18S rRNA gene amplification were 1391f (forward primer) and EukBR (reverse primer) with template specific regions 5'-GTACACACCGCCCGTC-3' and 3'-GATCCTTCTGCAGGTTCACCTAC-5' respectively (Amaral-Zettler *et al*., 2009). The PCR reaction mix was set to cycling parameters with initial denaturing at 98°C for 5 minutes (1 cycle), then 23 cycles of 98°C for 45 seconds, 57°C for 30 seconds, 72°C for 45 seconds, and final elongation at 72°C for 5 minutes.

The resultant PCR products were run in 1.5% SeaKim® LE agarose gel (Lonza) against DNA marker (Lambda/*Pst*I) at 100V for an hour. The agarose gel was stained with SYBR™ Safe DNA gel stain (Thermo Fischer Scientific) and TAE buffer containing 40 mM Tris base (Sigma Aldrich), 1 mM EDTA (Sigma Aldrich), and 20 mM glacial acetic acid (Sigma Aldrich) was used for gel electrophoresis. The PCR products were visualised on a UV transilluminator (ChemiDocTM XRS+, Bio-Rad), and the DNA bands corresponding to the correct amplification target were cut out of the agarose gel for purification. The amplicon products were purified using FavorPrep™ Gel

Purification Mini kit (Favorgen Biotech Corp) as per the manufacturer's instructions and 40 µL of PCR/amplicon product was eluted. The purified amplicon libraries were barcoded in preparation of multiplexing using Nextera XT V2 adaptors (Illumina) as per the instructions and further purified using AmPure XP beads (Beckman Coulter). A Qubit dsDNA HS assay (Invitrogen) was used to quantify the prepared amplicon libraries using Qubit™ 4 Fluorometer and the pooled libraries were validated using the Bioanalyser HS dsDNA chip (Agilent) on the Bioanalyser 2100 platform (Agilent). The libraries underwent sequencing on a Miseq platform (Illumina) with Nextera v3 600 cycle (Illumina) kit and sequence data was uploaded to the NCBI Sequence Read Archive (SRA) Bioproject number: PRJNA612973.

2.8 Computational analysis

2.8.1 Amplicon libraries

Data curation was performed on each of the datasets using Mothur software version 1.41.3 (Schloss *et al*., 2009) (Appendix 4). Sequence reads containing ambiguous nucleotides and those that were shorter than 300 bp and longer than 580 bp in bacterial datasets (16S rRNA) were removed while those that were shorter than 100 bp and longer than 350 bp were removed from the Eukaryotic dataset (18S rRNA). The sequences were classified against the Silva reference database (version 132) (Quast *et al.,* 2013) and non-target sequences were removed. Chimeras were detected using VSearch (Rognes *et al*., 2016) and together with singletons were removed from the dataset.

In order to compare sequence reads fairly, the 16S rRNA sequences from Sundays Estuary were subsampled down to 7800 and the combined dataset from Swartkops Estuary and point sources were subsampled to 8497 while the 18S rRNA dataset was subsampled down to 10 695. Operational taxonomic units were identified at a distance of 0.03 subsequent to clustering using the furthest neighbour algorithm (Schloss *et al.,* 2009).

Rarefaction curves, Observed, Chao1, and InvSimpson diversity indices were generated at the species level to assess diversity, richness, and evenness of bacteria (16S rRNA) and Eukaryotes (18S rRNA) in estuaries and point sources. The rarefaction curves were constructed in Microsoft excel while the diversity indices were calculated in the R platform using Phyloseq (Cardoso *et al*., 2015). Primer software (v7) was used to generate PCoA and NMDS plots based on Bray Curtis (Stuij, 2018), subsequent to square root transformation. Primer (v7) was also used for statistical analysis of similarities (ANOSIM) of the bacterioplankton and eukaryotic community profiles between sampling sites based on the Bray Curtis dissimilarity, as well as on Spearman rank correlation (Hummert, 2018) to assess the impacts of environmental variables on biological community composition and diversity. Distance-Based multivariate Linear Model analysis (DistLM) assessed the impact of individual variables on biological variation using Akaike Information Criterion (AICc) (Brewer *et al.*, 2016).

Bacterial and eukaryotic community composition and abundance were conducted at the family level using Microsoft excel. All the bacterial and eukaryotic families that were less than 1% of the total number of sequences in the datasets were grouped as minor families. The top 10 most numerically dominant OTUs in each dataset (Sundays 16S rDNA, Sundays 18S rDNA, Swartkops and point sources 16S rDNA) were selected from each sample, and averages of the replicates were calculated. The bacterial OTUs (16S rDNA) were standardised and presented in heatmaps using Primer software (v7) while the eukaryotic OTUs (18S rDNA) graph was constructed using excel. Bacterial OTUs that displayed interesting trends were subjected to BLASTn analysis (Altschul *et* al., 1990) against sequences in the GenBank nr/nt database and GenBank RefSeq_rna sequences online databases for identification at genus/species level while the eukaryotes OTUs were aligned against the GenBank nr/nt database.

Potential waterborne pathogenic bacteria were identified from the literature and their corresponding 16S rRNA sequences were extracted from the NCBI reference database to create a customised pathogen database. The Swartkops Estuary and associated water sources datasets were then analysed using standalone BLAST (Tao, 2010) against the newly created pathogen

database. Only sequence reads with >99% sequence identity to reference sequences in the pathogen database were retained and heatmaps were constructed using the Primer software (v7).

CHAPTER 3: MICROBIAL COMMUNITIES ALONG THE LENGTH OF THE AGRICULTURALLY INFLUENCED SUNDAYS ESTUARY

3.1 Physico-chemical characteristics of Sundays Estuary

Environmental variables were measured through the water column from eight sampling sites along the length of Sundays Estuary at ~0.5 m depth intervals (Figure 3.1 and Appendix 1: Table S1). Salinity and dissolved oxygen showed a noticeable trend along the estuary. As expected, salinity decreased from the mouth to the head of the estuary with higher salinities recorded in the 2019 sampling.

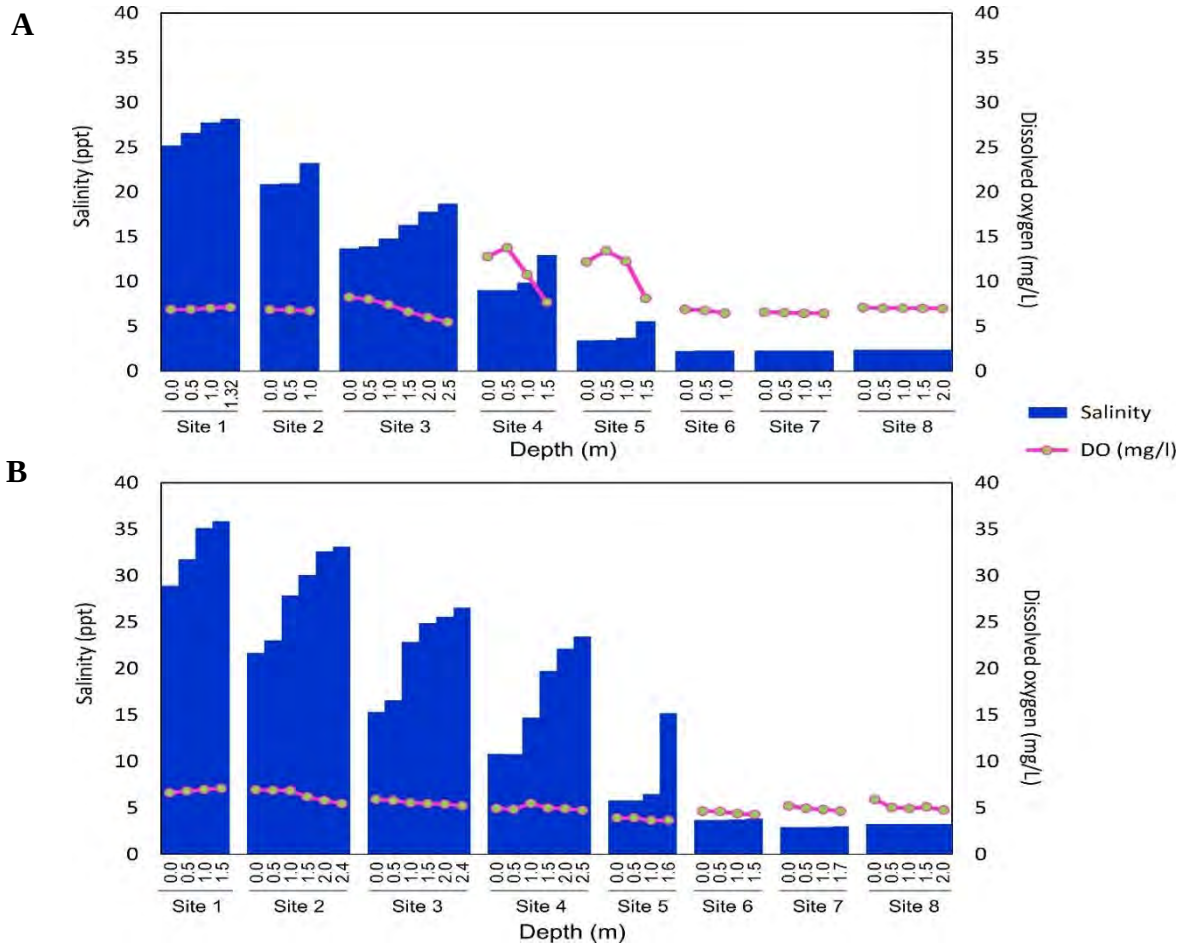

Figure 3.1: Dissolved oxygen (DO) and salinity gradient recorded at different depths through the water column from eight sampling sites along the length of Sundays Estuary in 2018 **(A)** and 2019 **(B)** during the sampling done in the spring season.

The middle and lower reaches of the estuary were mesohaline (5.0 – 18 ppt) and polyhaline (18 -30 ppt) respectively, while the upper reaches had riverine water inflow and were therefore oligohaline (< 5.0 ppt). Even though the tidal coefficient was higher in 2018 than in 2019 at the time of sampling, overall salinity was highest in 2019 ranging between 2.96 and 35.9 ppt while it ranged between 2.27 and 28.19 ppt in 2018. The water was at slack tide when sampling the first four sites from the mouth of the estuary in 2018, while water movement was slightly higher and was heading towards a slack tide when sampling the mouth of the estuary in 2019 thus resulting in the higher salinity. A distinct halocline was observed in the lower and middle reaches of the estuary with surface waters exhibiting lower salinities than that of the bottom waters. The lower reaches of the estuary were polyhaline, while in 2019, the incoming tides pushed farther into the estuary recording salinity of more than 30 ppt at the middle and bottom of site 1 and bottom of site 2 (Figure 3.1). The head of the estuary was dominated by freshwater with a relatively constant salinity gradient. Whilst salinity decreased from the mouth to the head of the estuary, dissolved oxygen (DO) levels changed inconsistently, ranging between 5.5 - 13.81 mg/L and 3.66 - 7.11 mg/L in 2018 and 2019 respectively. A drastic increase in DO content was observed on the surface and subsurface of sites 4 and 5 during sampling in 2018 which may be indicative of increased photosynthetic activity due to increases in resident phytoplankton abundances. Whilst salinity and DO noticeably changed throughout the water column of the estuary, water temperature and pH were relatively stable (Appendix 1, Table S1).

The impact of agricultural input in Sundays Estuary was evidenced by the high levels of nutrients at the upper reaches closer to the agricultural farms (Figure 3.2). The highest level of total oxidised nitrogen (TOxN) was recorded in 2018 and ranged between 0.52 – 81.6 µmol/L while it ranged between 3.95 – 30.6 µmol/L in 2019. Though the upper estuary had higher levels of TOxN in 2018, the lower and middle reaches recorded higher concentrations of TOxN in 2019 than in 2018.

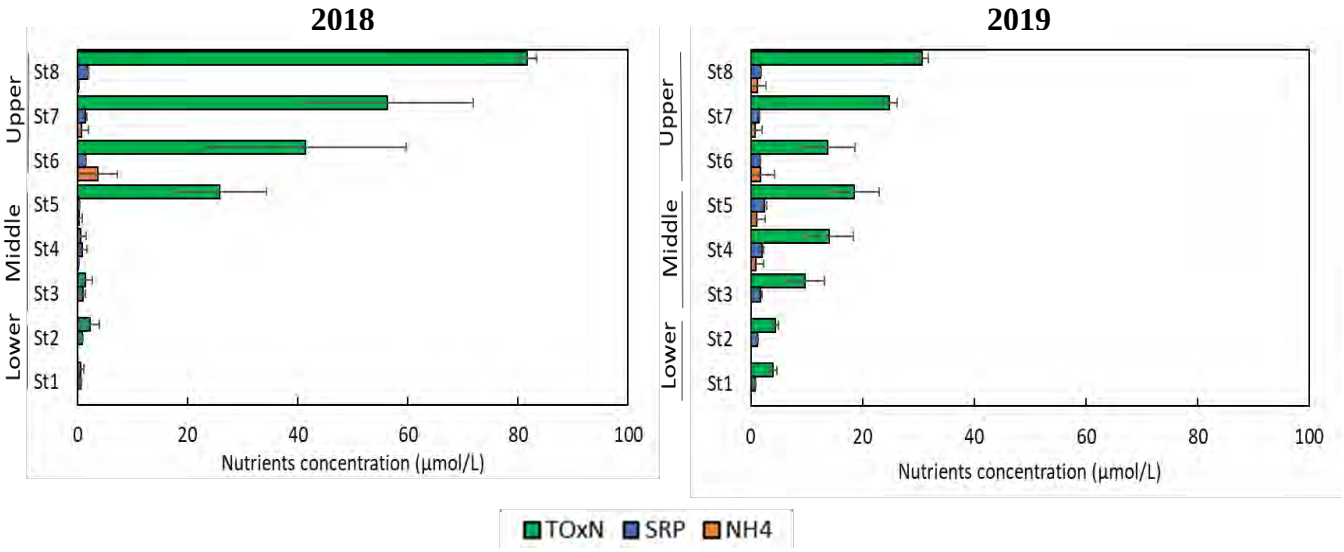

Figure 3.2: Average concentrations of total oxidised nitrogen (TOxN), Ammonium (NH_4) and soluble reactive phosphorus (SRP) measured through the water column from eight sampling sites along the length of Sundays Estuary in 2018 and 2019 during the spring sampling.

The data show that the sites dominated with freshwater at the upper reaches exceeded the normal threshold concentration of inorganic nitrogen (in TOxN) in freshwater aquatic ecosystems (> 500 µmol/L) in 2018, as outlined by the Department Of Water Affairs And Forestry, 1996, and the middle reaches with brackish water also exceeded the normal threshold concentration of inorganic nitrogen (trophic conditions) in estuarine ecosystems (≥ 100 µmo/L) in 2019 as outlined by Lemley *et al*. (2015). This is likely due to lower tides in 2018 and higher tides in 2019. Unlike TOxN, there were negligible changes in ammonium (NH_4) and soluble reactive phosphorus (SRP) concentrations between the two sampling periods (Figure 3.2). NH_4 had the lowest concentration ranging between 0.09 – 3.7 µmol/L and 0.8 – 1.7 µmol/L in 2018 and 2019 respectively, and it was not detected at the lower reaches or site 3 during both sampling periods. SRP ranged between 0.3 – 1.9 µmol/L and 0.7 – 2.4 µmol/L in 2018 and 2019 respectively (Figure 3.2). Even though SRP exhibited negligible changes along the estuary, the amount of inorganic phosphorus (in SRP) in the water column of Sundays Estuary at the upper reaches exceeded the normal threshold for trophic conditions in freshwater (>5 µmol/L) and estuarine water (≥10 µmol/L) in both years.

3.2 Phytoplankton Biomass

Phytoplankton biomass and composition were determined using chl-*a* (as a proxy for biomass and/or productivity), as well as microscopic morphological identification and phytoplankton cell counts (Figure 3.3).

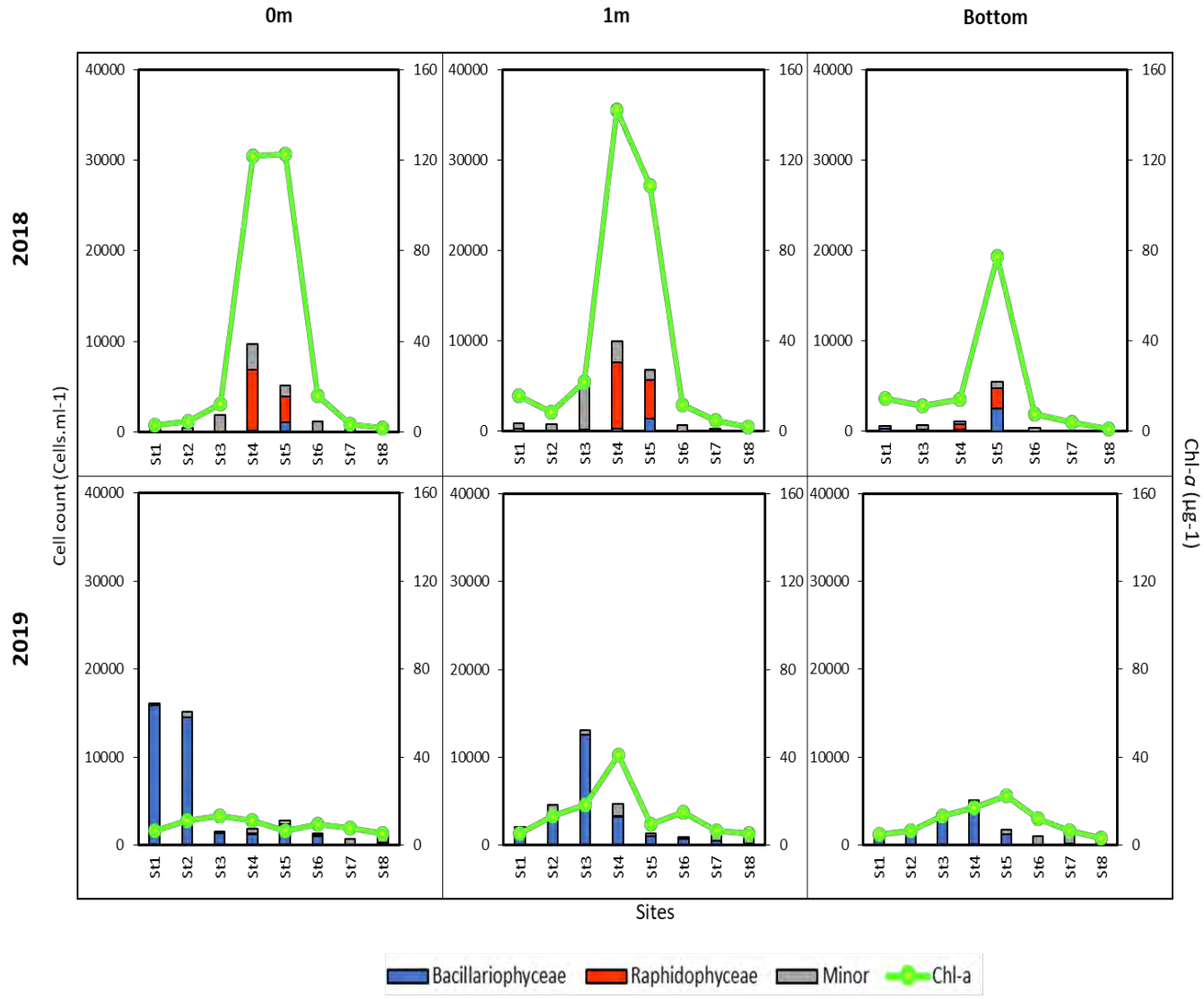

Figure 3.3: Biomass of dominant phytoplankton in the surface (0m), middle (1m) and bottom of the water column in Sundays Estuary during sampling in 2018 and 2019 in the spring season . Phytoplankton cells were classified according to algal functional groups. (Minor Phytoplankton: groups of microalgae which had few cell counts including *Dinophyceae*, *Euglenophyceae*, *Cryptophyceae*, *Chlorodendrophyceae*, *Cyanophyceae* and *Chrysophyceae*).

Phytoplankton biomass varied along the length of Sundays Estuary as well as exhibiting differences between the samples collected in 2018 and 2019 (Figure 3.3). As indicated by the increased levels of chl-*a,* the estuary had high phytoplankton-derived primary productivity in 2018 which corresponded with the high nutrient levels detected (Figure 3.2). The water surface and at ~1 m depth of the water column at the middle reaches (sites 4 and 5) were dominated by *Raphidophyceae* with biomass of >1 696 cells.mL^{-1} which qualifies it to be a bloom (Matcher *et al*., 2021). In contrast, the bottom of the water column was dominated by *Bacillariophyceae* with the highest cell count recorded (2 581 cells.mL^{-1}) at site 5 in 2018, however, this site also had a high cell count of *Raphidophyceae* (2 212 cells.mL^{-1}). The phytoplankton cell counts at these sites coincide with high chl-*a* concentrations of >60 µg.L^{-1} and can be classified as hypereutrophic (Lemley *et al*.,2015). The morphological detection of *Bacillariophyceae* and *Raphidophyceae* was corroborated in the molecular analysis of the 16S rRNA gene (Figure 3.6).

Although *Dinophyceae* and *Chlorodendrophyceae* were non-dominant in the estuary, they were in high cell counts from the lower to the middle reaches during sampling in 2018 except for the middle and bottom of the water column of site 1 which had higher cell counts of *Cryptophyceae* (Appendix 1, Figure S1). *Cryptophyceae* were also abundant at the upper estuary. Whilst chl-*a* is typically used as a measure of phytoplankton biomass, it did not correlate well with phytoplankton cell numbers in this study in 2019. There were high *Bacillariophyceae* cell counts (> 1, 000 cells.mL^{-1}) at most sites along the length of the estuary in 2019 particularly from the lower to the middle estuary (Figure 3.3). *Cyanophyceae* had high relative cell counts at the water surface of site 8 and ~1m and bottom of sites 7 and 8 while *Cryptophyceae* had high relative cell counts at different sites of the estuary and *Chlorodendrophyceae* were in high relative abundance at ~1m and bottom of site 4 (Appendix 1, Figure S1) in 2019. The morphological detection of *Cyanophyceae* (i.e. *Cyanobacteria*) (Appendix 1; Figure S1) was corroborated in the molecular analysis of the 16S rRNA gene (Figure 3.11).

3.3. EUKARYOTE COMMUNITY PROFILES AND DIVERSITY

Metabarcoding of the 18S rRNA gene was used to investigate the eukaryote communities within the water column along the length of the Sundays Estuary. A total of 1 005 330 sequences remained in the dataset after subsampling and data curation.

3.3.1 Eukaryote Alpha Diversity

The plotted graphs in this chapter represent all eukaryotes that were identified by the 18S rDNA sequence analysis in Sundays Estuary, which constitutes phytoplankton, fungi, and animals. Rarefaction curves were plotted to verify if sampling of eukaryotes was done to completion (Figure 3.4).

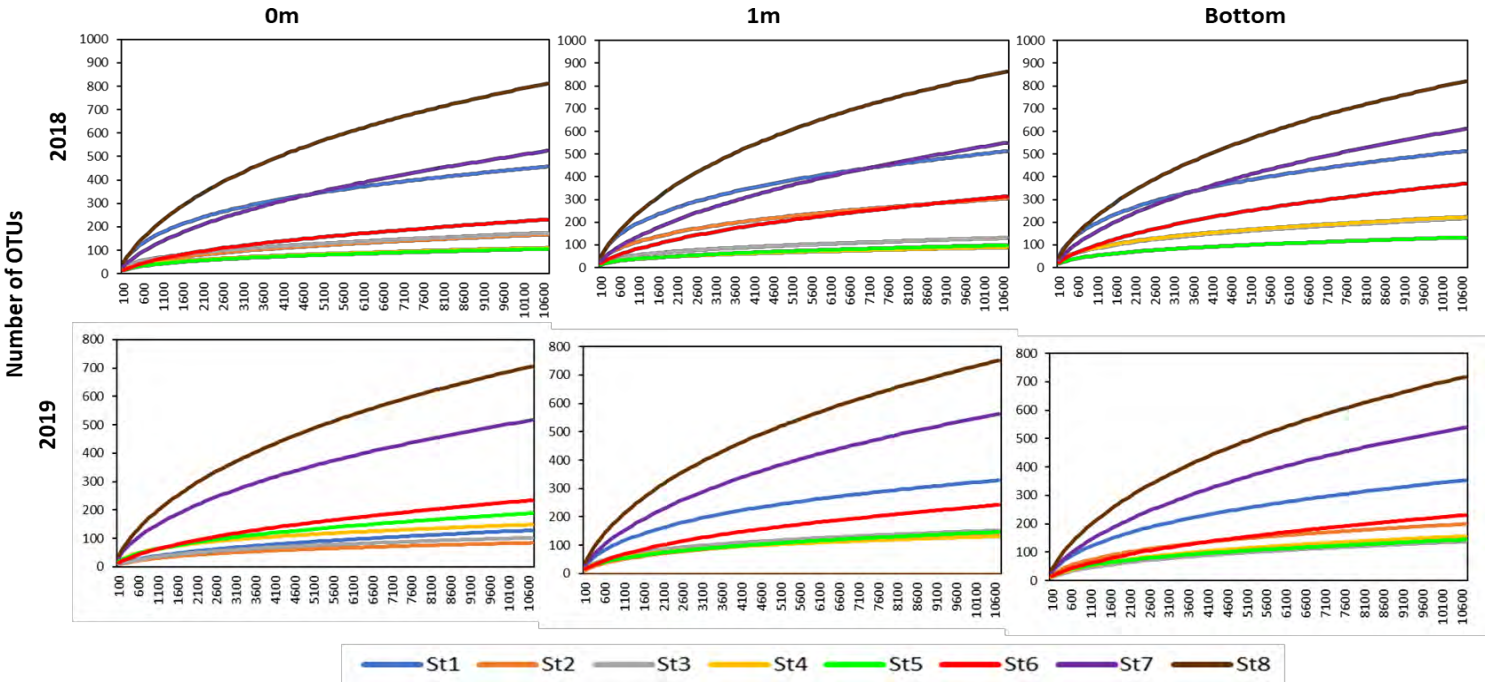

Figure 3.4: Rarefaction curves of eukaryotic OTUs sampled from the water surface (0m), middle (1m) and bottom of the water column along the length of Sundays Estuary at the time of sampling in the spring season in 2018/2019. Subsampling was done at 10 695 sequence reads during data curation and OTUs were generated at a distance of 0.03. The estuary was shallow at site 2 with water column of only 1m in depth in 2018.

Rarefaction curves as a representation of sequencing depth of sampling, indicate that sites 2 to 6 were almost sampled to completion with rare species included, while the rarefaction curves for the upper reaches suggested that some of the more rarely occurring OTUs may have been missed (Figure 3.4). This is not surprising as these sites also exhibited the greatest richness (Figure 3.5)

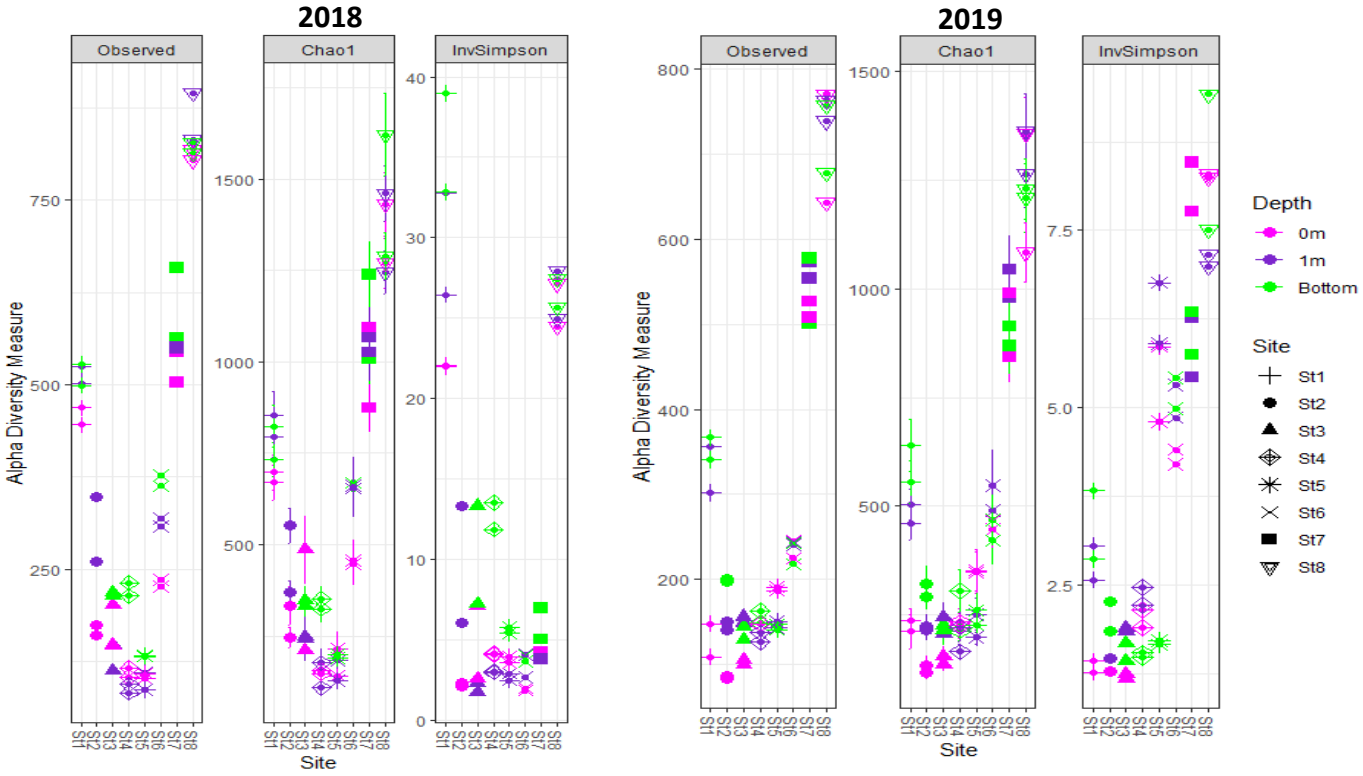

Figure 3.5: Species richness (Observed and Chao1) and species richness and diversity (InvSimpson) at the water surface (0m), middle (1m) and bottom of the water column in the Sundays Estuary at the time of sampling in spring 2018 and 2019. OTUs were generated at a distance of 0.03.

At the time of sampling in both 2018 and 2019, Chao1 estimates reflected an increase in species richness at site 1 followed by decreased species richness at sites 2-6 (Figure 3.5). The observed increase in species richness at site 1 is likely due to the incoming marine water carrying associated marine eukaryotes which then mix with the specialized estuarine eukaryotic communities. At sites 7 and 8, which are freshwater dominated (Figure 3.1), very high levels of species richness were observed (Figure 3.5).

Diversity is dependent on species richness and evenness of OTUs (Kim *et al.,*2017). When comparing eukaryote diversity between the two years, the estuary had a remarkably higher diversity in 2018 than in 2019 (Figure 3.5, note the axes) which is likely due to differences in the evenness of distribution of the OTUs (since their relevant richness (i.e. Chao1) indices were similar). This may be a reflection of the *Bacillariophyceae* bloom which was noted at the same time of sampling in 2019 (Figure 3.3, Figure 3.6).

3.3.2. Taxonomic classification of 18S rRNA libraries

A diverse group of eukaryotes classified at the taxon level of the family was identified along the length of Sundays Estuary, particularly at the time of sampling in 2018 (Figure 3.6), which is also reflected in Figure 3.5. The 18S rRNA sequence dataset was primarily represented by phytoplankton while only two families of animals (*Oligotrichia* and *Maxillopoda*) and one family of fungi (*Rhizophydiales*) were dominant. There was a noticeable difference in the presence and abundance of eukaryotes between the sites and between the two years (Figure 3.6). *Oligotrichia* (ciliates) contributed to >60% of the sequences in the dataset at site 2 in 2018 and were noted in sites 2-5 in both years (Figure 3.6). Reads assigned to the taxa *Maxillopoda* and *Rhizophydiales* were restricted to the upper reaches and were more prevalent in 2019 (Figure 3.6).

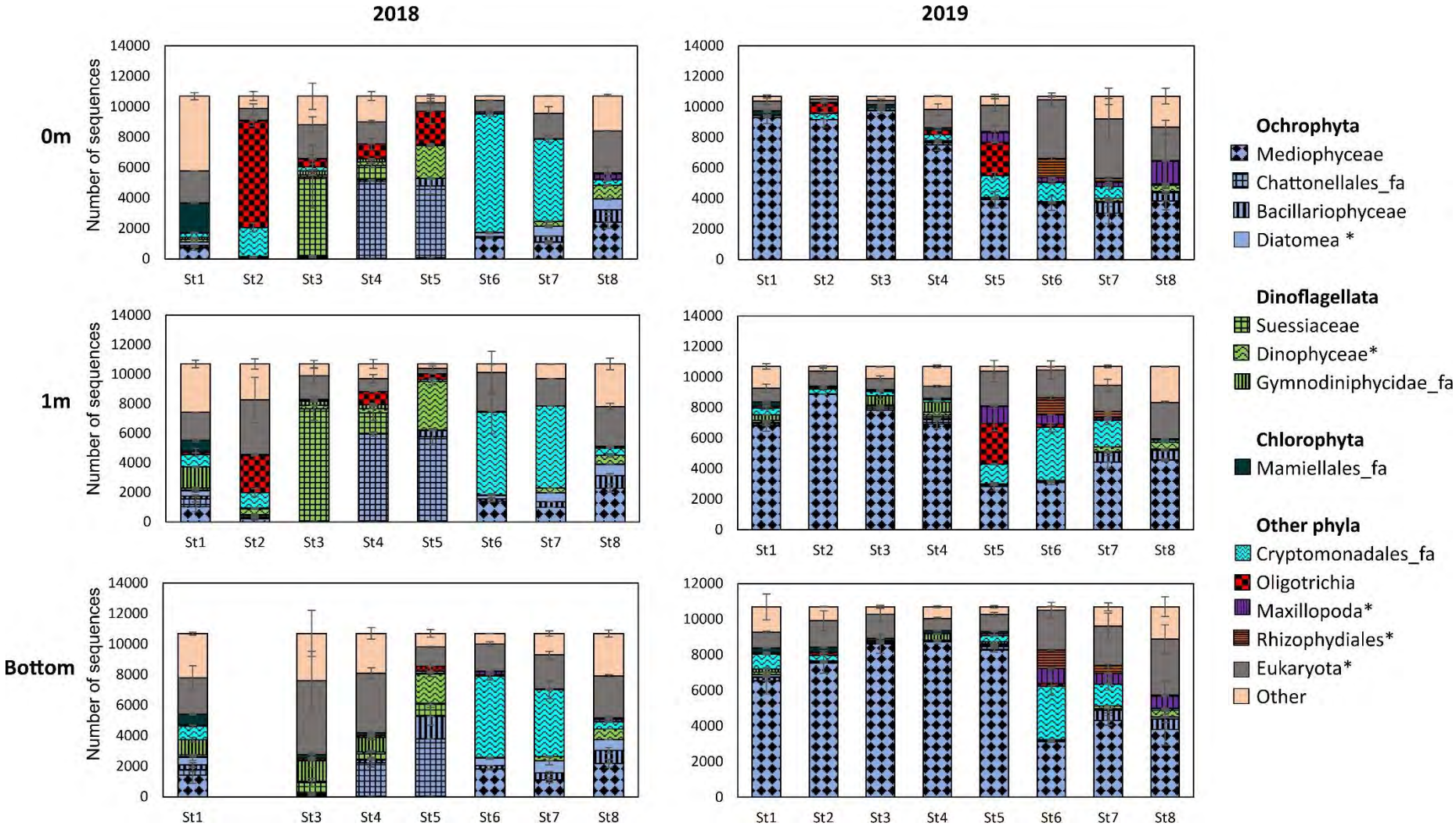

Figure 3.6: Phylogenetic characterization of the eukaryotes communities at the taxon level of family in the surface (A), middle (B) and bottom (C) of the water column along the length of Sundays Estuary in 2018 and 2019. The estuary was shallow at site 2 with water column of only 1m in depth in 2018. Error bars represent standard deviation of each family taxon in each sampling site (n=2). Unclassified families are labelled with an asterix (*). Lower reaches of the estuary (sites 1, 2), Middle reaches (sites 3, 4, 5), Upper reaches (sites 6, 7, 8).

With respect to the phytoplankton community, the middle of the estuary had high relative abundances of the members of *Suessiaceae, Dinophyceae,* and *Chattonellales_fa* at the time of sampling in 2018, that were in low relative abundance at other sites of the estuary and at the time of sampling in 2019, while *Cryptomonadales_fa* which contributed 16.7% of the total sequences dominated at sites 6 and 7 and were low in relative abundance at other sampling sites of the estuary. The *Chattonellales_fa* dominated the water surface and 1m of the water column at sites 4 and 5 and bottom of site 5, correlating with high dissolved oxygen, *Raphidophyceae* cell counts, and chl-*a* concentrations (Figures 3.1 and 3.3). Site 3 had high relative abundances of *Suessiaceae* particularly at the middle of the water column with low relative abundance at the bottom of the water column, while *Dinophyceae* were relatively high in abundance at site 5. Site 1 was noted as having a high relative abundance of *Mamiellales_fa* (marine picoplanktonic algae) and a greater proportion of non-dominant phyla (other phyla) at the time of sampling in 2018 (Figure 3.6) which may have contributed to a high diversity of eukaryotes communities for this site (Figure 3.5). Whilst the estuary was dominated by diatoms belonging to the taxon *Mediophyceae* (Bacillariophyta) at the time of sampling in 2019, contributing 57% of the total sequences (Figure 3.6), this taxon was relatively low in abundance at the time of sampling in 2018 accounting for <7.5% of the total sequences. Members of this family decreased in relative abundance as one progressed up the estuary in 2019. The upper reaches seemed to provide a perfect niche for members of *Cryptomonadales_fa*, as they were also identified in high relative abundance in 2018 (Figure 3.6).

3.3.3. Dominant Eukaryotic OTUs

The top ten most dominant eukaryote OTUs at genus/species level are detailed in Appendix 1 (Figure S2) and the OTUs of particular interest/dominance summarised in Figure 3.7 and Table 3.1. The OTUs were significantly different along the length of the estuary in both 2018 and 2019 with a p-value (global) of 0.001 (Appendix 1, Table S2). Dominant OTUs in Sundays Estuary (Figure 3.7), showed a similar pattern of abundance with their respective families plotted in Figure 3.6. As was observed in Figure 3.6, a lower diversity was also exhibited at the genus/species level during sampling in 2019. In both 2018 and 2019, an algal bloom was observed at the time of sampling with 2018 exhibiting a *Raphidophyceae* bloom and

a *Bacillariophyceae* bloom occuring in 2019 (Figure 3.3). Blooms are typically characterised monophyletic representation of algae and this can be clearly seen by the dominance of OTU53 and OTU109 (Figure 3.7) which had sequence identities to *Heterosigma akashiwo* (100% identity) and *Cylotella* (>98% identity) respectively (Table 3.1). A second *Cyclotella* species (OTU103) was found in the upper reaches in 2019 although this did not correlate with the morphological cell count data (Figure 3.3) which suggests that this species was in the pre-bloom stage.

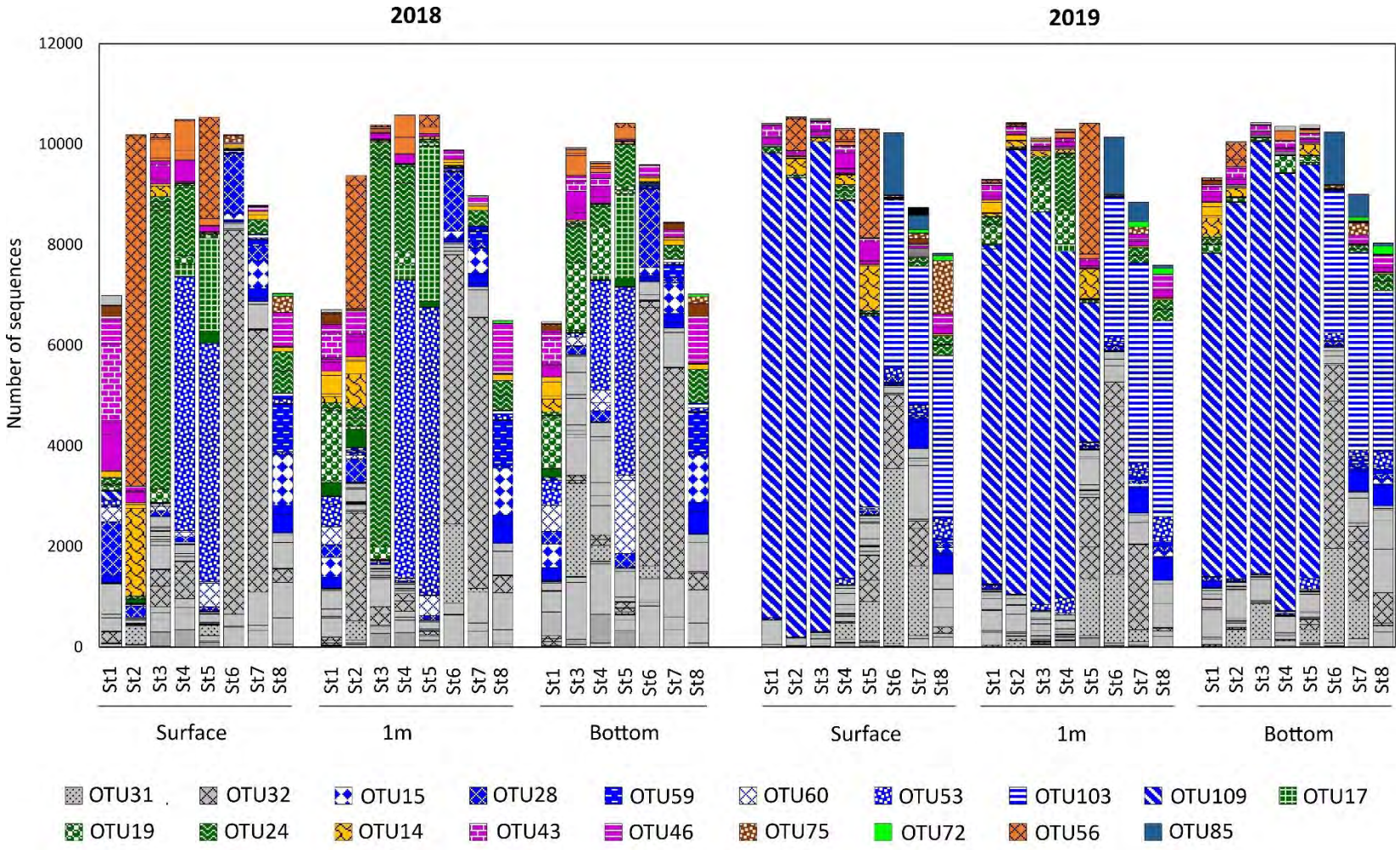

Figure 3.7: The top 10 most dominant eukaryotes OTUs sampled during spring season at the surface, 1m and bottom of the water column of Sundays Estuary in 2018/2019. OTUs were generated at distance of 0.03. Site 2 was shallow with depth of 1m in 2018. The lower reaches of the estuary are sites 1 and 2, middle reaches; sites 3, 4, 5, and upper reaches; sites 6, 7, 8. (The grey color represent unclassified eukaryotes, Blue – Ochrophyta, Green - Dinoflagellata, Orange – Cilliophora, Purple – Chlorophyta, Yellow – Cryptomonadales)

Table 3.1: Blast match results of eukaryotes OTUs of interest at genus/species level against the GenBank database and Silva_v132 reference database

OTUs	Closest Match against Silva v132	Match against nr/nt GenBank		
		Accession Number	Identity match %	Closest Taxonomic match in NCBI
OTU14	*Cryptomonadales*_unclassified	FJ884690.1	100%	Cryptophyta sp.
OTU15	*Mediophyceae*_unclassified	DQ093370.1	100%	*Stephanodiscus hantzschii*
OTU17	*Dinophyceae*_unclassified	KC336918.1	97.56%	uncultured eukaryote
OTU19	*Dinophyceae*_unclassified	FN669510.1	97.01%	*Gyrodinium dominans*
OTU24	*Suessiaceae*_unclassified	KJ763207.1	100%	uncultured eukaryote
OTU28	*Bacillariophytina*_unclassified	KT347147.1	97.60%	*Conticribra weissflogiopsis*
OTU31	Eukaryota_unclassified	KP768155.1	92.26%	*Pseudodiaptomus marinus*
OTU32	Eukaryota_unclassified	FR874777.1	98.19%	Uncultured picoeukaryote
OTU43	*Mamiellales*_unclassified	FR874768.1	100%	Uncultured picoeukaryote
OTU46	*Ulvales*_fa_unclassified	MN070070.1	98.22%	*Ulva flexuosa*
OTU53	*Heterosigma*	AY788934.1	100%	*Heterosigma akashiwo*
OTU56	*Oligotrichia*_unclassified	EF100412.1	100%	Uncultured eukaryote
OTU59	*Mediophyceae*_unclassified	EF585582.1	98.80%	*Conticribra weissflogii*
OTU60	Diatomea_unclassified	KT347147.1	98.8%	*Conticribra weissflogiopsis*
		DQ093368.1		*Thalassiosira tumida*
OTU75	Maxillopoda_unclassified	GU070895.1	100%	Invertebrate
OTU85	*Kappamycetaceae*_ge	EU162640.1	99.38%	uncultured Chytridiomycota
OTU103	*Cyclotella*	KY364697.1	98.20%	*Cyclotella cryptica*
OTU109	*Cyclotella*	KT346253.1	98.80%	uncultured eukaryote

Aside from OTU53 (*Heterosigma akashiwo*) and the two *Cyclotella* species (OTU103 and OTU109), other phytoplankton prevalent in the Sundays Estuary included *Ochrophyta* (OTU15, OTU28, OTU59, OTU60), *Dinoflagellata* (OTU17, OTU19, OTU24), *Chlorophyta* (OTU53 and OTU46) and *Cryptomonadales* (OTU14) (Figure 3.7). The upper reaches of the estuary had high relative abundances of OTU15 (100% sequence identity to *Stephanodiscus hantzschii*), OTU28, and OTU59 with sequence similarity to *Conticribra weissflogiopsis* and OTU46 with 98% sequence similarity to *Ulva flexuosa* during sampling in 2018 while they were low in relative abundance in 2019 (Figure 3.7, Table 3.1). While OTU15 was numerically abundant through the water column of sites 6 and 7, OTU28 was in high relative abundance through the water column of site 6, and OTU59 and OTU46 were high in relative abundance through the water column of site 8. OTU15 was also numerically abundant at the middle and bottom of site 1. OTU24 and OTU15 were abundant at sites 3-5 in 2018 although they were present in both years of sampling. The

lower reaches had a high relative abundance of OTU14 (with 100% sequence identity to *Crypytophyta* sp.) in 2018. OTU43 was present through the water column of site 1 and numerically abundant at the water surface, while OTU14 was numerically abundant at the water surface of site 2 and decreased down the water column. By contrast, OTU14 was high in relative abundance at the water surface and middle of the water column of site 5 as well as the bottom of site 1 during sampling in 2019, although it was noted in other sites of the estuary. OTU19 (97% sequence identity to *Gyrodinium dominans*) was numerically abundant at the middle of the water column of site 1 and bottom of sites 1 - 3 in 2018, while it was present at the lower and middle reaches of the estuary, though in low relative abundance during sampling in 2019.

With respect to the *Oligotrichia* (ciliates), in 2018, OTU56 was most abundant at the water surface of site 2 and middle of the water column, while it shifted in abundance to the water surface and middle of the water column of site 5 during sampling in 2019 (Figure 3.7, Table 3.1). OTU85 (*Chytridiomycota*), which also did not share significant sequence similarity to any genera in the reference database, was identified in the upper reaches of the estuary in 2019, particularly at site 6, and was absent or in low relative abundances in 2018. OTU75, which is an unknown copepod of the *Maxillopoda* family, was distinctively abundant at the water surface of site 8 while it was absent/ low in relative abundance at other sites of the estuary and during sampling in 2018.

3.4. BACTERIAL COMMUNITY PROFILES AND DIVERSITY

3.4.1. Bacterial Alpha Diversity

To ascertain that the sequencing depth of bacterial communities from the water column of the eight sampling sites in Sundays Estuary was done to completion, bacterial OTUs generated at a distance of 0.03 were presented in rarefaction curves (Figure 3.8). A total of 733 200 sequences remained in the dataset after data curation and subsampling. All the rarefaction curves were reaching/nearing asymptote which verifies that the majority of OTUs were captured and sampling of bacteria was done

to completion, and increasing sampling depth would not have provided significant further information for the majority of the bacterial community profiles in these samples. It is important to keep in mind that although the sequencing depth was sufficient to detect most of the OTUs, not all of the very rare species were represented in the curated dataset.

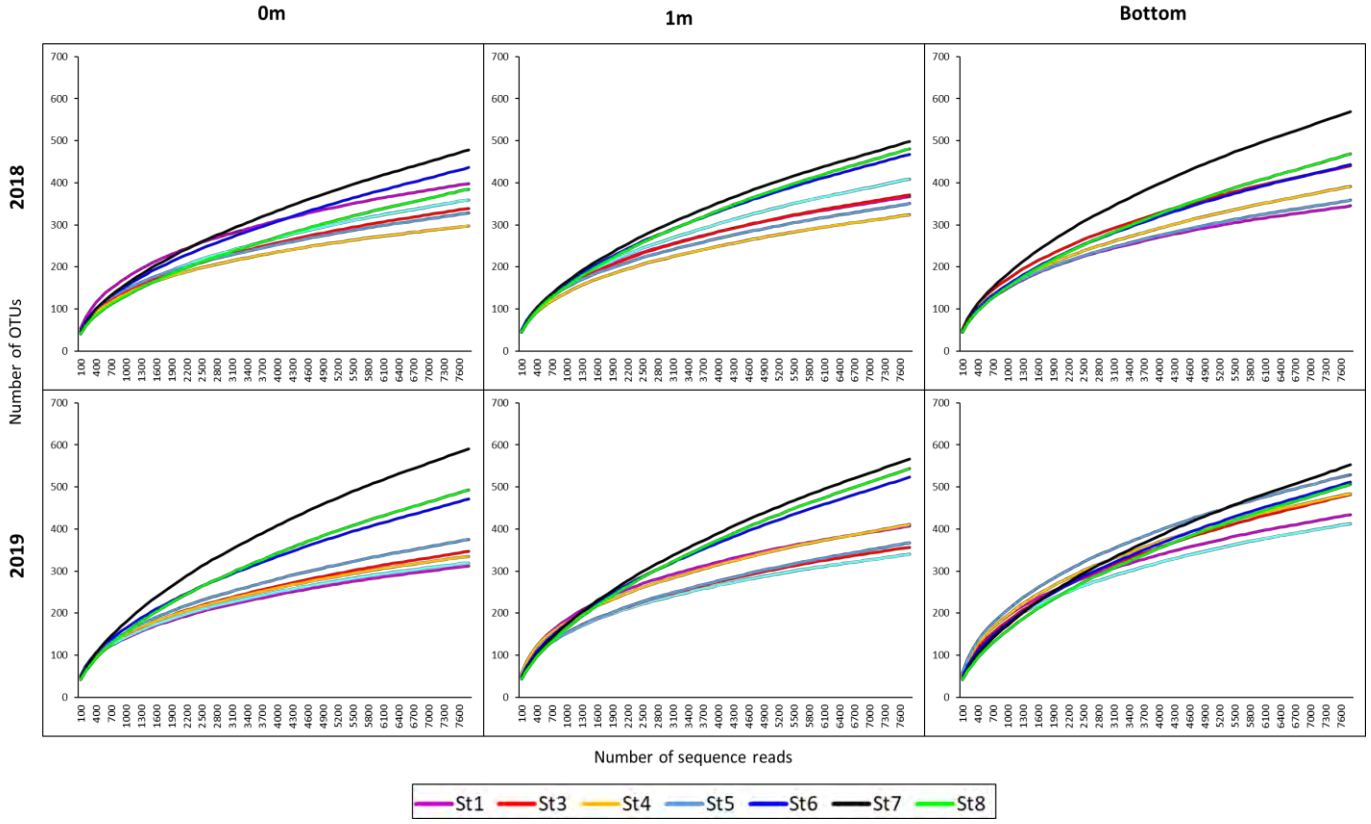

Figure 3.8: Rarefaction curves of bacterial OTUs at the water surface (0m), middle (1m) and bottom of the water column along the length of the Sundays Estuary. Samples were subsampled at 7800 sequence reads during data curation and OTUs were generated at a distance of 0.03.

A distinction in bacterial OTUs richness, as presented by Observed and Chao1, and OTU diversity (InvSimpson) was observed between sampling sites (Figure 3.9).

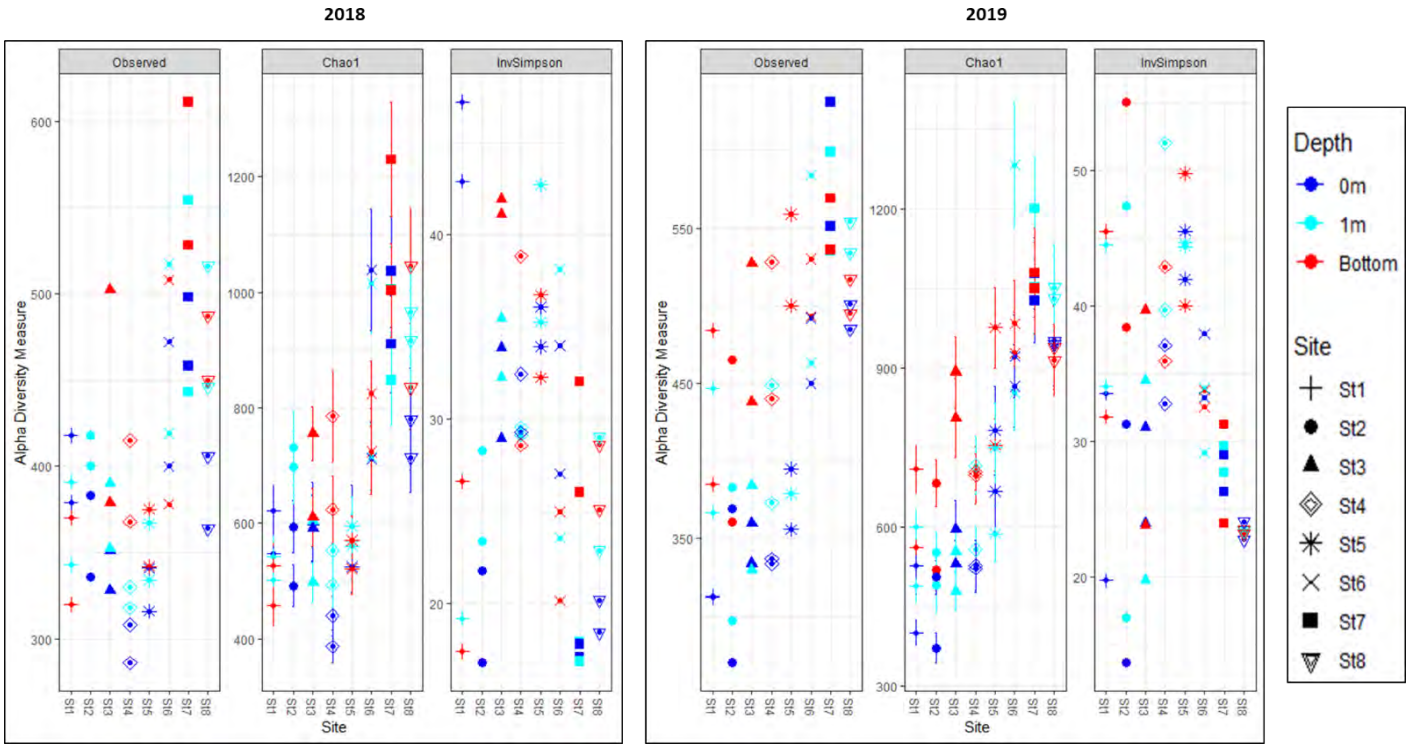

Figure 3.9: Alpha diversity indices representing species richness (Observed and Chao1) and species richness and evenness (InvSimpson) of the bacterial communities in the water column at the surface (0m), middle (1m) and bottom of sites 1 to 8 along the Sundays Estuary in 2018 and 2019 during sampling in the spring season.

Chao1 predicts richness of both the observed OTUs and the unsequenced rare OTUs. As observed OTUs may miss the very rare species within the bacterial community, it is therefore not surprising that Chao1 had higher values than the observed number of OTUs in the dataset (Figure 3.9). This corroborates what was observed in the rarefaction curves in that the dominant/abundant OTUs were captured in the current datasets whilst some of the very rare OTUs were not represented. The samples collected in 2018 and 2019 both had high species richness. Species richness varied along the length of the estuary as well as through the water column. The upper estuary had higher species richness which decreased towards the lower estuary during both sampling periods. The surface waters of site 4 in 2018, which had algal blooms at the time of sampling, had the least species richness. In 2019, the bottom of the water column at site 3 had higher species richness compared to the samples collected from the surface and middle of the water column of the same site, while site 8 had higher species richness at ~1m depth. In general, high species richness was observed at sites with high nutrient levels. However, bacterial populations at these sites were unevenly distributed as indicated by the InvSimpson diversity index, which means that

there was low diversity compared to the lower and middle reaches of the estuary. Even if the bacterial OTUs were unsubsampled, a similar trend in bacterial richness and diversity would have shown (Appendix 1, Figure S3).

3.4.2. Bacterial Beta Diversity

The plotted Principal Coordinate Analysis (PCoA) graphs explain the variation and the environmental factors influencing bacterial community profile variation at different sampling sites along the length of Sundays Estuary in 2018 and 2019 (Figure 3.10). Over ~77% of the variation observed in the bacterial communities along the length of the estuary is represented by these PCoA plots (Figure 3.10, see axes).

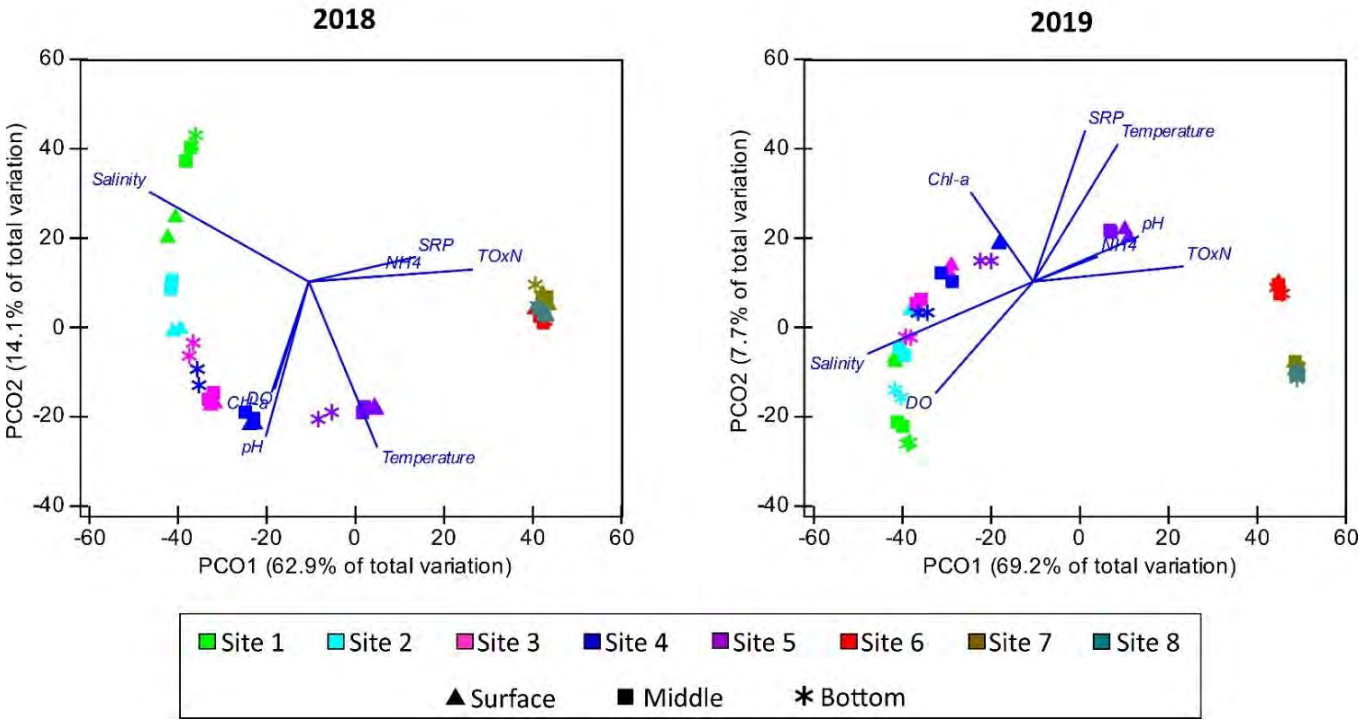

Figure 3.10: Principal Coordinate Analysis (PCoA) illustrating dis(similarity) between bacterial community profiles sampled at different sites through the water column and environmental variables driving OTUs compositional variation along the length of Sundays Estuary in 2018 and 2019. PCoA was run on Bray Curtis similarity derived from the Square Root Transformed abundance and OTUs were computed at a distance of 0.03.

Bacterial OTUs composition varied along the length of the estuary and was influenced by environmental conditions. Statistical analysis (Spearman rank correlation) showed a positive correlation between bacterial population profiles and the physico-chemical variables in both 2018 and 2019 (Rho = ~0.5, p = 0.001) (Appendix 1, Table S5). Since DistLM analysis indicated that salinity accounted for 61.58% and 67.64% of the variation in bacterial community composition in 2018 and 2019 respectively (Appendix 1, Table S6), sampling sites were thus classed as oligohaline, mesohaline, and polyhaline for conducting ANOSIM analyses. Oligohaline water occurred in the upper reaches of the estuary (sites 6, 7, and 8) where due to the freshwater inflow, salinity was < 5 ppt. Polyhaline waters occurred closest to the marine environment and are classified as having salinities of between 18 and 30 ppt whilst mesohaline (salinity values of 5 – 18 ppt) occurred in a narrow range between the polyhaline and oligohaline waters in the Sundays Estuary. ANOSIM analysis of the bacterial community composition showed a significant difference (global test, p = 0.001, R-value = 0.815 to 0.835) between marine, polyhaline, mesohaline, and oligohaline samples (Appendix 1: Table S4).

In addition to salinity, bacterial distribution patterns correlated significantly with all other environmental variables that were measured along the water column in both years (Spearman rank correlation, $p < 0.05$), with BEST (statistical analysis of correlation) highlighting dissolved oxygen, temperature, TOxN, NH_4 and chl-*a* as the best variables that correlated with the distribution patterns in 2018, while salinity gradient and TOxN correlated best with distribution of bacterial communities during sampling in 2019 (Appendix 1, Table S6). Sites at the upper reaches were closer to the agricultural farms in the catchment area, therefore it is not surprising that bacterial community composition had a close relationship with nutrients (TOxN, SRP, and NH_4) which were probably a result of runoff of fertilizers from agricultural farms (Figure 3.10).

3.4.2.1. Bacterial Taxonomic Classification

As was seen with OTUs variation along the estuary (Figure 3.10), Sundays Estuary had diverse bacterial communities at the taxon level of family (Figure 3.11). Dominant bacterial families (with the number of sequence reads >1% of the total number of sequences) identified in the water column along the length

of Sundays Estuary showed a shift in terms of population distribution patterns and abundance between the two years.

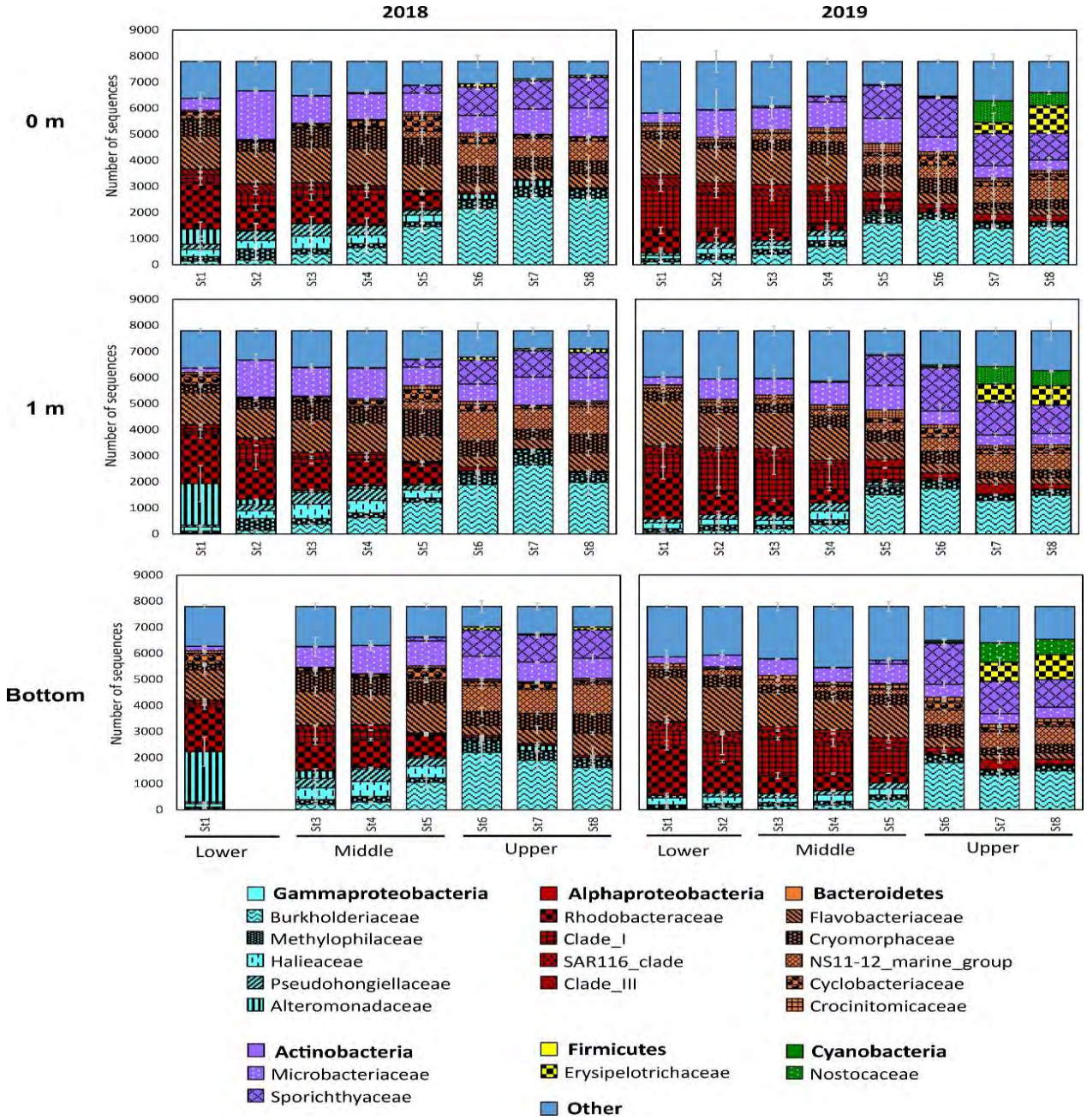

Figure 3.11: Phylogenetic characterization of the bacterial communities at the taxon level of family in the surface (A), middle (B) and bottom (C) of the water column along Sundays Estuary in 2018 and 2019. The estuary was shallow at site 2 with water column of only 1m in depth. Error bars represent standard deviation of each family taxon in each sampling site (n=2).

The phylum Proteobacteria, comprised mostly of reads belonging to the classes Gammaproteobacteria and Alphaproteobacteria*,* was predominant through the water column contributing 37.3% of the total sequences with Gammaproteobacteria increasing in relative abundance from the mouth to the upper reaches and vice-versa for Alphaproteobacteria (Figure 3.11). Within the Gammaproteobacteria, members of the *Burkholderiaceae* family were the most dominant at the upper reaches and decreased in relative abundance towards the lower reaches, while the middle reaches had diverse bacterial families which also decreased to the lower reaches. *Burkholderiaceae* and *Methylophilaceae*, together with some bacterial families from other phyla including *Sporichthyaceae* (Actinobacteria), NS-11-12_marine_group (Bacteroidetes), *Erysipelotrichaceae* (Firmicutes), and *Nostocaceae* (Cyanobacteria) were in high relative abundance at the upper reaches where there was inflow from riverine water and high nutrient inputs (Figure 3.11 and Figure 3.2). *Sporichthyaceae* extended in high relative abundance further downstream (to site 5) in 2019 at the water surface and 1 m of the water column than that which was found in 2018 (Figure 3.11). Furthermore, the two years exhibited a distinction in bacterial abundance as indicated by Clade_I, *Nostocaceae,* and *Erysipelotrichaceae* which were noticeably higher in relative abundances during sampling in 2019. Some bacterial taxa, including all families belonging to Gammaproteobacteria, *Rhodobacteraceae* (Alphaproteobacteria), *Cryomorphaceae* (Bacteroidetes), and NS11-12 marine group (Bacteroidetes), exhibited increased relative abundances in 2018 when the estuary had high levels of nutrients and *Raphidophyceae* blooms, as opposed to samples collected in 2019.

Flavobacteriaceae, the family to which reads within the phylum Bacteroidetes were frequently assigned to (40% of Bacteroidetes reads), and bacterial families of the Alphaproteobacteria class decreased in relative abundances from the lower to the upper reaches of the estuary, and the proportion of the bacterial community assigned as *Rhodobacteriaceae* noticeably increased towards the bottom of the water column in 2019. *Cryomorphaceae, Halieceae* (Gammaproteobacteria), and *Pseudohongiellaceae* (Gammaproteobacteria) were also identified along the length of the estuary at the time of sampling but were predominantly found in the datasets from the middle reaches of the estuary in 2018 during *Raphidophyceae* blooms (Figure 3.11).

Notably, the *Alteromonadaceae* observed at site 1 in 2018 increased in relative abundances from the surface down to the bottom of the water column which correlates perfectly with salinity gradient and coincides with a relative increase in chl-*a* concentration (Figure 3.11 and Figure 3.3). High salinity at the lower reaches of the estuary in 2019 correlated with a high relative abundance of Clade_I which belongs to the order SAR11_clade. SAR11_Clade also has brackish and freshwater clades such as Clade_III which were identified at the middle and upper reaches of the estuary in 2019. A larger proportion of the datasets in 2019 were grouped within the minor phyla than was the case for 2018 (Figure 3.11) which correlates well with the increased diversity indices observed in 2019 (Figure 3.9).

3.4.2.2 Dominant bacterial OTUs

The abundance and diversity of OTUs within the bacterial communities corroborates the variation observed in Figure 3.10 and Figure 3.11. The top ten most dominant OTUs from the surface, middle, and bottom of the water column of each sampling site were identified, split into their respective phyla, and presented in heatmaps (Figure 3.12A & B). The OTUs highlighted in the heatmaps were subjected to BLASTn analysis against the NCBI nr/nt database consisting of a nucleotide collection from sequences from GenBank, EMBL, DDBJ, PDB, RefSeq, as well as against the NCBI 16S ribosomal database (RefSeq) which is formed from curated type strains (Appendix 1: Table S7).

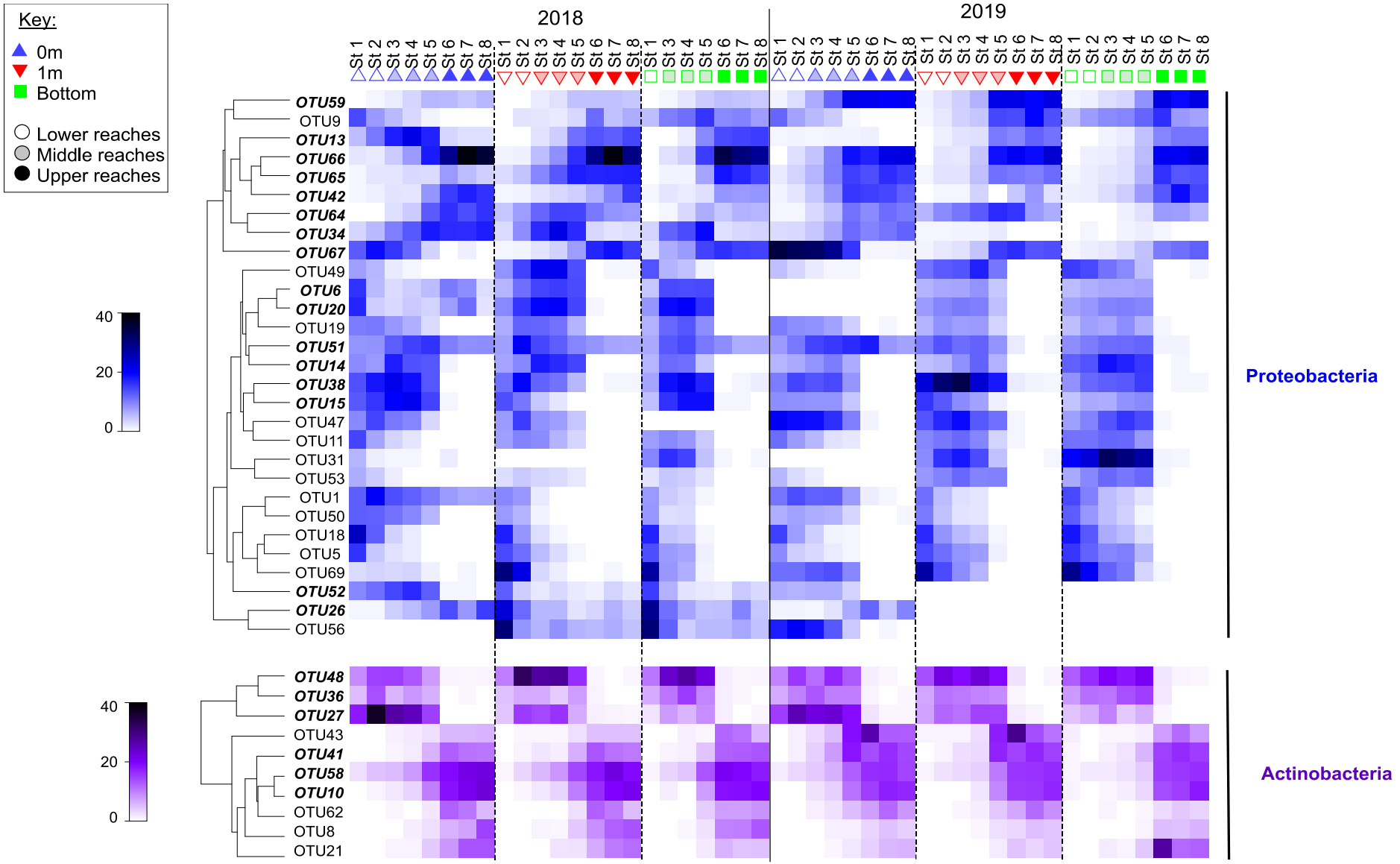

Figure 3.12 A: Heatmap of the top 10 most dominant OTUs assigned to the phyla Proteobacteria and Actinobacteria collected from the surface, middle and bottom of the water column, at each sampling site along the length of Sundays Estuary during the 2018 and 2019 spring sampling. (n=2). OTUs were clustered at distance of 0.03 and data was transformed to square root and standardised. OTUs of interest are highlighted in bold and italics. The estuary was shallow at site 2 with water column of only 1m in depth.

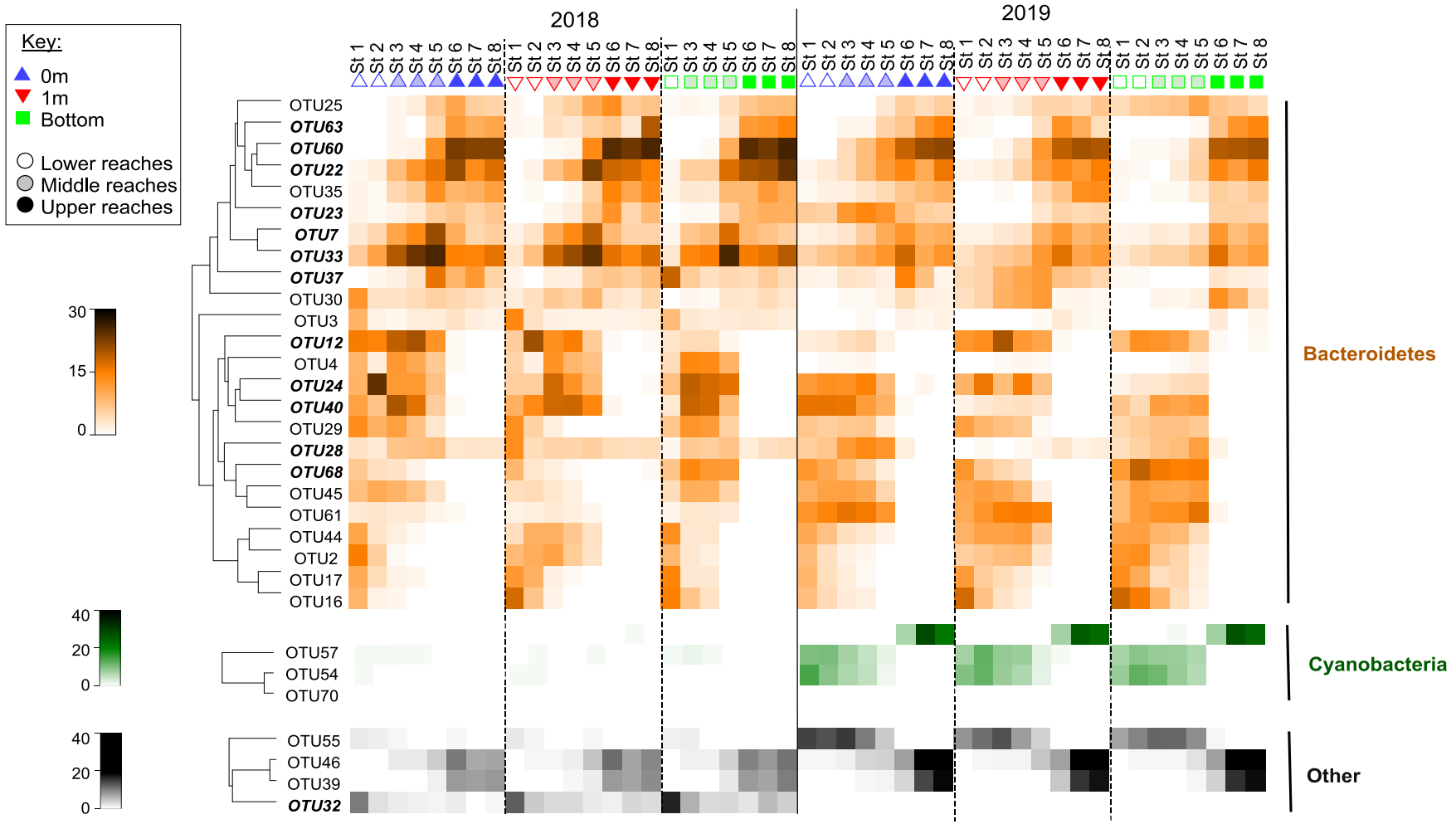

Figure 3.12 B: Heatmap of the top 10 most dominant OTUs assigned to the phyla Bacteroidetes, Cyanobacteria and Other phyla (Firmicutes and unclassified bacteria) collected from the surface, middle and bottom of the water column at each sampling site, along the length of Sundays Estuary during the 2018 and 2019 spring sampling. (n = 2). OTUs were clustered at distance of 0.03 and data was transformed to square root and standardised. OTUs of interest are highlighted in bold and italics. The estuary was shallow at site 2 with the water column of only 1m in depth.

In depth analysis of the top ten most dominant OTUs at each sample interval and depth (Figure 3.12 A & B) revealed that these OTUs accounted for 60 to 75% of the total number of sequences in each sample dataset. A clear trend was observed in the OTU distribution patterns from the lower reaches to the upper reaches of the estuary with many OTUs occurring primarily only in the upper reaches or only in the lower reaches. However, the middle reaches had an abundance of dominant OTUs which were either a representation of the lower or upper reaches or a mixture of both. Of interest were OTU38, OTU15, and OTU14 (Proteobacteria) and OTU33, OTU12, OTU7, and OTU40 (Bacteroidetes) which appeared to be more prevalent at the middle reaches than either the upper or lower reaches. While this trend is not always as striking in both years, it still holds true (Figure 3.12 A & B, Appendix 1: Table S7).

OTU51 (Proteobacteria) with sequence identity to RS62_marine_group as identified against the Silva database (version 132), was noted throughout the water column and along the entire length of the estuary during sampling in both years, with reduced relative abundances in the upper reaches at 1 m and bottom of the water column in 2019 sampling (Figure 3.12A, Appendix 1: Table S7). In contrast, OTU20 and OTU6, which exhibited >98% sequence similarity to *Rheinheimera pleormorphica,* were represented in the surface waters along the entire length of the estuary in 2018 but not in 2019, although these two OTUs were found in the lower and middle reaches at the depths of 1 m and the bottom of the water column in both years. OTU67 (99.5% sequence identity to Candidatus Pelagibacteria) was the most abundant within the Proteobacteria OTUs at the water surface of the lower to the middle reaches in 2019 and was also identified at 1 m and bottom of the water column in the upper reaches in both years.

In addition to the previously mentioned Proteobacteria OTUs which were numerically abundant at the middle reaches in 2018 during the *Raphidophyceae* blooms (namely OTU14, 38, and 15), OTU13 (>98.3% sequence identity to *Idiomarina aestuarii*) and OTU52 (97.32% sequence identity to *Donghicola eburneus*) were in high relative abundance at the water surface of the middle reaches in 2018 (Figure 3.12A, Appendix 1: Table S7). OTU13 was also numerically abundant at 1 m and bottom of the water column in the upper reaches and interestingly, it was in very low abundances at the water surface during sampling in 2019, while OTU52 was identified in abundance at the middle and bottom of the water

column at site 1 and was absent/low in relative abundance at the middle and bottom of the water column during sampling in 2019. OTU26 (97.57% sequence identity to *Hydrogenophaga taeniospiralis*) was in high relative abundance at the water surfaces of sites 6 and 8 and middle and bottom of the water column in the lower reaches in samples collected in 2018. This OTU was absent along the entire length of the estuary at 1 m depth and bottom of the water column (Figure 3.12A). OTU66 (Proteobacteria), which also showed high sequence identity to *Hydrogenophaga taeniospiralis,* was found in high relative abundances through the water column in the upper reaches particularly during sampling in 2018 (Figure 3.12A, Appendix 1: Table S7). OTU59, OTU34, OTU64, which exhibited >98% sequence identity to isolates from the MWH-UniP1_aquatic_group of the *Burkholderiaceae* family, along with OTU65 (99% sequence identity to Candidatus Fonsibacter ubiquis) and OTU42 (98.5% sequence identity to Candidatus _methylopumilus) were also in high relative abundance at the upper reaches and decreased in relative abundance towards the mouth of the estuary. However, OTU59 was more abundant in 2019 than in 2018, and OTU34 was also found in increased abundance at 1 m depth and bottom of the water column in the middle reaches in 2018 while it was in very low relative abundance along the length of the estuary at 1 m depth and bottom of the water column during sampling in 2019. OTU64 was numerically abundant at the middle reaches at 1 m of the water column in 2018 and the middle of the water column of sites 5 and 6 during sampling in 2019. Of interest to note, dominant OTUs at the upper reaches of the estuary were mostly members of the *Burkholderiaceae* family of the order *Betaproteobacteriales* (Figure 3.12A, Appendix 1: Table S7).

The length of the estuary had a definite split between Actinobacteria OTUs in the lower to the middle reaches and that of the upper reaches (Figure 3.12A). OTUs that were prevalent at the lower to the middle reaches of the estuary irrespective of the year of sampling include OTU48, OTU27, and OTU36. OTU27 (with 98.8% sequence identity to Candidatus Aquiluna rubra*)* was numerically abundant particularly at the water surface of site 2 in 2018 while OTU48 was abundant from sites 2 to 4 at the middle and bottom of the water column during sampling in 2018 and extended in abundance further to site 5 in 2019 (Figure 3.12A, Appendix 1: Table S7). All other dominant OTUs of the phylum Actinobacteria were prevalent at the upper reaches. OTU10 (99% sequence identity to Candidatus_Planktophila

sulfonica) and OTU58 (98.8% sequence similarity to Candidatus_Limnoluna rubra) were the most abundant at the upper reaches within this phylum particularly in samples collected in 2018.

The most dominant Bacteroidetes OTU at the upper estuary in 2018 and 2019 was OTU60 with sequence similarity to NS11-12_marine_group which showed a similar pattern of abundance at the family level (Figure 3.12B, Appendix 1: Table S7, Figure 3.11). OTU63, which also had sequence similarity to NS11-12_marine_group, was also prevalent at the upper reaches and decreased in relative abundance towards the lower estuary. OTU23 and OTU68 also had sequence similarities to NS11-12_marine_group. OTU23 had a higher number of reads assigned to it at the upper estuary during sampling in both years with the exception of the water surface in 2019, where it was numerically abundant at the middle reaches. OTU68 decreased in relative abundance from the lower to the upper reaches in the water surface and middle of the water column while it was in increased abundance from the lower to the middle reaches at the bottom of the water column during both sampling periods. OTU22, which did not share significant sequence similarity to any genera in the NCBI reference database but was identified as a member of the *Cryomorphaceae* against the Silva v132 database, was also numerically abundant at the upper reaches of the estuary particularly during sampling in 2018. Although OTU12, which also had sequence similarity to members of the family *Cryomorphaceae*, was in high relative abundance at the water surface of the middle reaches in samples collected in 2018, it was also present in high relative abundance at the lower reaches in the water surface and middle of the water column and from the lower to the middle reaches at 1 m and bottom of the water column during sampling in 2019. While OTU28 occurred along the length of the estuary in 2018, it was numerically abundant in the water surface of the middle reaches during sampling in 2019. In addition to the previously mentioned OTUs that were numerically abundant at the middle reaches during sampling in 2018 (namely OTU33, OTU7, OTU12, OTU40), OTU24 with sequence similarity to NS3a-marine_group was also numerically abundant at the middle reaches in 2018 showing a closer association with *Raphidophyceae* blooms. However, this OTU together with OTU40 (NS3a-marine_group*)* was in high relative abundance at the surface of the lower reaches in 2019 where there were *Bacillariophyceae* blooms.

Whilst Cyanobacteria were recorded in low abundance during sampling in 2018, OTU57 (98.3% sequence identity to *Dolichospermum flos-aquae*) was numerically abundant at the upper reaches during sampling in 2019, and OTU54 and OTU70 were present at the lower to the middle reaches of the estuary (Figure 3.12B, Appendix 1: Table S7). In contrast to the pattern of abundance of the phylum Firmicutes in Figure 3.11, the only dominant OTU of this phylum identified in the estuary (OTU32 with 99.5% sequence similarity to *Exiguobacterium himgiriensis),* was in high relative abundance through the water column at site 1 during sampling in 2018*.* OTU46 and OTU39 did not share significant sequence similarity with any genera in the reference database and were numerically abundant at sites 7 and 8 during sampling in 2019.

3.5. Discussion

Sundays Estuary has previously been reported as an agriculturally impacted estuary in South Africa with high levels of nutrients entering the estuary from farming activities, in particular citrus farms, in the catchment area (Scharler *et al*., 1997; Lemley *et al*., 2017). This corresponds to the results in this study with TOxN and SRP concentrations exceeding the recommended threshold levels (Lemley *et al.,*2015) (Figure 3.2). Whilst TOxN was the main source of nutrient pollution in both years, higher levels were observed in 2018 as opposed to 2019. SRP concentrations were typically higher in the upper and middle reaches of the estuary with higher amounts detected in 2019. The poor water quality in the Sundays Estuary was evident by its eutrophic state with the occurrence of algal blooms in the estuary. The morphological analysis of phytoplankton identified *Raphidophyceae* as the cause of the blooms that occurred during sampling in 2018. A closer examination of members of this taxon to species level indicated high cell counts of *Heterosigma akashiwo* which was identified as the main cause of the blooms in 2018 while *Bacillariophyceae* blooms were identified with high *Cyclotella* cell counts noted during sampling in 2019. Blooms impact the physico-chemical conditions in the estuary. In literature, during the peak of algal blooms, the physico-chemical conditions of the water are typically altered including decreased dissolved oxygen, depleted nutrients, and increased pH (Lemley *et al*., 2020). In this study, however, dissolved oxygen increased at *H. akashiwo* bloom sites and, together with pH, were in the same range with the results by Lemley *et al*. (2020) (Figure 3.1, Appendix 1; Table S1). The uptake of

nutrients differs between phytoplankton species (Baek *et al*., 2020) and *H. akashiwo* is one of the phytoplankton species that rapidly takes up nutrients (Lemley *et al*., 2018a). This was clearly evident in this study with a rapid decline in nutrient concentrations, especially that of TOxN, at bloom sites while nutrient concentrations decreased moderately during sampling in 2019 when members of the *Bacillariophyceae* dominated the estuary (Figure 3.2, 3.6).

Phytoplankton are sensitive to environmental perturbations and are therefore commonly used as indicators of environmental pollution in estuaries (Paerl *et al*., 2006). Algae grow best with readily available sources of nitrogen and ammonium (Admiraal *et al*., 1987; Underwood *et al*., 1998), as such, high levels of growth in a nutrient polluted estuary where nitrogen levels exceeded the recommended threshold levels is likely. Phytoplankton blooms in estuaries are linked to increased freshwater inflow which carries nutrients loads from the catchment (Froneman & Vorwerk, 2013). This was evident with high levels of TOxN at the upper estuary adjacent to citrus farms which indicates an influx of fertilizer-derived nitrogen. The middle reaches of the Sundays Estuary were supersaturated with dissolved oxygen and exhibited high chl-*a* concentrations (Figures 3.1, 3.3) during sampling in 2018, particularly at sites 4 and 5. This was probably due to high relative abundances of members of the family *Chattonellales* (Figure 3.6) which was almost exclusively represented by reads assigned to OTU53 with 100% sequence similarity to *Heterosigma akashiwo* (Figure 3.7, Table 3.1). This species has a dense concentration of chloroplasts which increases photosynthetic activity (Lemley *et al*.,2020) hence high dissolved oxygen. *H. akashiwo* is frequently reported as forming harmful algal blooms in coastal water bodies and estuaries (Martínez *et al*., 2010; Dursun *et al*., 2016) and has also been observed to form recurring blooms in Sundays Estuary (Lemley *et al*., 2017; 2018a; 2020; Matcher *et al*., 2021). The morphological studies of the phytoplankton present during the 2018 sampling identified a bloom formed by cells belonging to the class *Raphidophyceae* (Figure 3.3). This concurs with the results obtained in the 18S rRNA metabarcoding dataset which identified large numbers of *H. akashiwo* which falls within the taxon *Raphidophyceae*. Lemley *et al*. (2018a, 2018b) highlighted high nutrient availability, ability to photosynthesize, high temperatures, and motility as factors influencing *H. akashiwo* blooms in Sundays Estuary. In addition, Martínez *et al*. (2010) highlighted mesohaline-polyhaline water conditions as one of the best growth conditions for *H. akashiwo* blooms, while results presented by Strom *et al*. (2013) showed high tolerance

of this phytoplankton species in a wide range of salinities including growth in freshwater. The measured variables in this study were within the favorable water conditions for *H. akashiwo* blooms at the time of sampling (~3 – 13 PSU and 22.3 – 23.5^{o}C).

Blooms are likely to impact species richness (Harlin, 1995) as was seen with low richness at bloom sites during sampling in both 2018 and 2019. *H. akashiwo* can tolerate some eukaryotes species (Shikata *et al*., 2008b), and suppress the growth of others with its toxins (Livingston, 2007; Lemley *et al*., 2018b, 2020). Low species richness may also be due to the ability of *H. akashiwo* to outcompete other eukaryotes as it can survive well in both low and high nutrient levels (Zhang *et al*., 2006). Despite the toxicity of harmful *H. akashiwo* blooms on fish and other aquatic animals (Khan *et al*., 1997; Suttle, 2000; Graham & Strom, 2010; Strom *et al*.,2013) and its ability to suppress zooplankton growth (Kamiyama *et al*., 2000; Lemley *et al*., 2018a), these species have shown to be non-toxic to Dinoflagellates (Strom *et al*., 2013) and it is not surprising that the estuary had a high relative abundance of *Dinophyceae* during sampling in 2018 (Figure 3.6, Appendix 1: Figure S1). The effect of harmful algal blooms observed in this study concurs with the results in a study by (Livingston, 2007).

Bloom succession seems to occur frequently in Sundays Estuary (Kotsedi *et al*., 2012) as was observed in this study with changes in bloom composition between 2018 and 2019. In a long-term study conducted by Kotsedi *et al*. (2012), different phytoplankton groups such as green algae, Flagellates, Dinoflagellates, and diatoms caused blooms during different sampling seasons. Hilmer (1990) and Jerling (1994) also reported Dinoflagellate blooms in previous studies in Sundays Estuary. In this study, the *H. akashiwo* bloom in 2018 was succeeded by a bloom of OTU109 with sequence identity to *Cyclotella* in 2019 (Figure 3.7, Table 3.1). High water stratification or reduced mixing favors the growth of *Cyclotella* (Winder *et al*., 2009), which was observed during sampling in 2019 (Figure 3.1). A high relative abundance of reads assigned to OTU109 was identified at the lower to the middle reaches during sampling in 2019 (Figure 3.7, Table 3.1), and correlated with *Bacillariophyceae* blooms (Figure 3.3). Traditional taxonomic ranking of diatoms includes the *Coscinodiscophyceae* (centric diatoms), *Fragilariophyceae* (araphid pennate diatoms), and *Bacillariophyceae* (raphid pennate diatoms) (Round *et al*., 1990) and this was the

classification which was utilized in the morphological studies. However, a further study of these classes by Medlin (2014) which included genetic barcoding, has resulted in the amendment of the class *Bacillariophyceae* into two classes namely *Bacillariophyceae* (pennate diatoms) and *Mediophyceae* (bipolar centric diatoms and radial *Thalassiosirales* diatoms). This accounts for the apparent discrepancy between the morphological identification of the diatoms in the Sundays Estuary (Figure 3.3 and Figure 3.6) and the genetic identification of *Cyclotella* (*Mediophyceae*) (Figure 3.7). The 2019 results presented in this chapter concur with the results reported in 2008 where *Cyclotella* was one of the causes of blooms in Sundays Estuary (Kotsedi *et al*.,2012). Whilst OTU109 caused blooms at the lower to the middle reaches in 2019, OTU103 which also had sequence similarity to *Cyclotella* sp. was found in high relative abundance at the upper reaches at the time of sampling in 2019 (Figure 3.7, Table 3.1), however, microscopic cell counts did not indicate the presence of high numbers of *Bacillariophyceae* in the upper reaches of the Sundays Estuary (Figure 3.3). It must be kept in mind that data generated from amplicon libraries represent relative proportions of the target populations. Thus, if the overall number of eukaryotic cells was low within the water column, then the proportion of *Cyclotella* sequence reads relative to that of the total eukaryote community present would be inflated. In this instance, *Cyclotella* definitely forms a large proportion of the eukaryotic community in the upper reaches of the estuary, however, *Cyclotella* cells were not in sufficient numbers to be classified as a bloom in the upper reaches which is in contrast to the lower reaches (St1-4) where a clearly defined *Cyclotella* bloom was observed (Figure 3.3).

Diatoms play a central role in the primary production in aquatic systems and contribute to nutrient cycling such as silicon and carbon (Pinckney & Zingmark, 1993; Litchman *et al*., 2009). They are also involved in the biogeochemistry of nickel in aquatic ecosystems and its distribution in the water column (Twinning *et al*., 2012). As cells with larger silicified cell walls sink they become part of the food source for benthic organisms and bacteria (Deckere *et al*., 2001), and they produce extracellular polymeric substances which stabilise the sediments and reduces erosion (Decho, 2000). Besides the *Cyclotella* species, a diverse range of diatoms were identified within the Sundays Estuary including *Chaetoceros*, *Thalassiosira*, *Navicula*, *Conticribra*, and *Stephanodiscus hantzchii* (OTU47, OTU60, OTU65, OTU3, OTU4, OTU59, OTU28, and OTU15) (Figure 3.7, Table 3.1, Appendix 1: Figure S2A and Table S3). OTU15 which

was prevalent in the Sundays Estuary during the 2018 sampling (Figure 3.7, Table 3.1), showed 100% sequence identity to the corresponding 18S rRNA gene of *Stephanodiscus hantzschii* in the NCBI database. While *Stephanodiscus hantzschii* has been reported as forming part of the blooms in August 2008 in the middle to the upper reaches in Sundays Estuary (Kotsedi *et al*., 2012), it was not observed in numbers indicative of a bloom event in this study. This species is a harmful freshwater diatom that prefers high nutrient environments but can still survive in estuarine conditions (Baek *et al*., 2020) and can be used as an indicator of eutrophication in aquatic ecosystems (Kolmakov *et al*., 2002) including phosphorus (Reavie & Kireta, 2015), nitrogen and silicon (Jung *et al*., 2011). OTUs 28 and 59 (Figure 3.7) exhibited sequence similarities to isolates from the genus *Conticribra* (Table 3.1). This genus has shown to grow rapidly in a wide range of salinities including marine, brackish and riverine water, and has high nutritional value (König *et al*., 2014; Li *et al*., 2016; Stachura-Suchoples & Kulikovskiy, 2019) which makes it more susceptible to grazing hence their presence in low abundance during sampling in 2019 when the estuary had high relative abundance of *Maxillopoda* which can be potential grazers (Figure 3.6).

Some of the other phytoplankton that were in high relative abundance in the lower estuary during sampling had sequence similarity to marine diatoms (OTUs 66 and OTU107). These had sequence identity to *Tenuicylindrus* and *Minidiscus* (Appendix 1: Figure S2B, Table S3). OTU20 with sequence identity to *Paraphysomonas* sp. (Appendix 1: Figure S2B, Table S3) was also found at the lower reaches of the estuary. *Paraphysomonas* are heterotrophic nanoflagellates that can be found in high salinity environments (Lim *et al*., 1999; Ishigaki & Sleigh, 2001; Tophøj *et al*., 2018) and their cells are covered with siliceous scales (Scoble & Cavalier-Smith, 2013). Some phytoplankton such as *Scenedesmus* sp. (Chlorophyta) have high uptake rates of nutrients such as nitrates, ammonium, and phosphates and have been used to remove high levels of nutrients from wastewater treatment plants (Pham & Bui, 2020). For this reason, it can be used as a bioindicator of nutrient pollution. Although the estuary had higher TOxN in 2018 than in 2019, OTU26 with 99.4% sequence similarity to this genus was in high relative abundance at the upper reaches in 2019 (Appendix 1: Figure S2C; Table S3). *Wislouchiella* is also associated with nutrient-rich conditions in freshwater habitats (Ullah, 2019; Kulaš *et al.,* 2021) and can be an indicator of nutrient pollution hence a high relative abundance of OTU89 at the upper estuary more especially in 2018 (Appendix 1: Figure S2C, Table S3).

Ulva is macroalgae that grows in a wide variety of habitats including brackish and freshwater (Rybak *et al*., 2014). However, low salinity and eutrophic conditions aggravate the growth of a wide range of the Ulva species (Hofmann *et al*., 2010). OTU46 was the only dominant OTU of the *Ulva* genus that was identified in the estuary (Appendix 1: Figure S2C, Table S3). *Ulva* species occur in filamentous forms which can detach from their substrates and float on the water surface, thereby limiting light penetration for benthic and submerged communities. It was previously identified from Sundays Estuary (Prinsloo, 2012). *Mamiellales* are photosynthetic marine picoeukaryotes that can also be found in abundance in coastal waters (Not *et al*., 2004; Yau *et al*., 2015). Therefore, it is not surprising that they were identified in high relative abundance at the water surface of site 1 and decreased to the bottom of the water column as salinity increased (Figure 3.6).

Sundays Estuary had a higher relative abundance of *Cryptomonadales*_fa in 2018 than in 2019 at the upper reaches of the estuary (Figure 3.6). *Cryptomonadales* can survive in both marine and freshwater environments (Moore *et al*., 2012), so is not surprising that the *Cryptomonadales* OTU69 (100% sequence similarity to *Teleaulux amphioxeia*) was present in the lower and upper estuary (Appendix 1: Figure S2B, Table S3). This species can form red tides when in abundance and serves as a source of food for ciliates (Lee *et al*., 2019). Whilst *Dinophyceae* were abundant at the middle reaches during sampling in 2018, the 18S rDNA metabarcording identified a high relative abundance of members of this family at site 5 (Figure 3.6) whilethe morphological analysis identified this taxon at site 3 (Appendix 1, Figure S1). *Gyrodinium* species are ubiquitous Dinoflagellates that are found in abundance in coastal waters as heterotrophic grazers of red tides or harmful algal blooms (Jae & Hae, 2004; Lee *et al*., 2014; Kang *et al*., 2020). The occurrence of OTU19 (97.01% sequence identity to *Gyrodinium*) in high relative abundance during sampling in 2018 may be due to the availability of *H. akashiwo* (OTU56) and *Cyclotella* (OTU103 and OTU109) as a readily available source of food (Figure 3.7, Table 3.1).

Even though the estuary had high primary production which contributed greatly to species richness and diversity, other eukaryotes that were identified in the estuary could not be overlooked as they play a role in energy cycling. A study by Sutherland *et al*. (2013) on the morphological identification of

zooplankton in Sundays Estuary, highlighted the majority of copepods whose composition was influenced by temperature, salinity, and freshwater inflow. The 18S rDNA metabarcoding analysis conducted in this study identified a small number of Arthropods. The majority of these Arthropods together with those of the Rotifera and the Porifera, were identified in this study at low salinity sites with high nutrient levels (Figure 3.6, Appendix 1: Figure S2C, Table S3). OTU49 exhibited 100% sequence identity to an *Oithona* sp. and members of this genus are one of the most abundant planktonic copepods in oceans (Gallienne & Robins, 2001). *Oithona* sp. were identified in high relative abundances at high salinity sites (Appendix 1: Figure S2C, Table S3). This genus was also found in high salinity conditions in the freshwater deprived Kariega Estuary, South Africa (Froneman & Vorwerk, 2013). *Oithona* sp. feed on phytoplankton including ciliates, diatoms, Dinoflagellates as well as protozooplankton, and is consumed by other zooplankton (Wang *et al*., 2017; Cornwell *et al*., 2020). Copepods can control diatom biomass after a bloom event (Not *et al*., 2004), and according to the dataset generated in this study, members of the *Maxillopoda* family, including OTU99 and OTU116, were in high relative abundance in 2019 corresponding to the high relative abundance of diatoms (Figure 3.6; Appendix 1: Figure S2C, Table S3).

The single dominant OTU belonging to the phylum Porifera noted in this study was OTU72 which had 100% sequence similarity to the freshwater sponge *Ephydatia fluviatilis.* This species was found predominantly in the upper reaches of the estuary in 2019 (Appendix 1: Figure S2C, Table S3). Gaino & Rebora (2003) found diatoms in *Ephydatia fluviatilis* vacuoles which suggest that it feeds on diatoms. Its presence in high relative abundance in 2019 may have been triggered by the abundance of diatoms. Ciliophora responded positively to phosphorus and oxidised nitrogen in a study by (Chariton *et al*., 2015) but they were in abundance at sites with lower nutrient levels in this study (Appendix 1: Figure S2C, Table S3). The *Oligotrichia* are marine ciliates and are commonly found in plankton communities (Doherty *et al*., 2010). This taxon was identified in high relative abundance within the phylum *Ciliophora* in Sundays Estuary (Figure 3.6). OTU56 assigned to this family was identified in high relative abundance at site 2 of the estuary and site 5 of the estuary in 2019 (Figure 3.7, Table 3.1). The importance of planktonic ciliates is often overlooked despite the role they play in linking microbial and macrobial elements of the food web (Doherty *et al*., 2010). *Chytridiomycota* can be associated with organic matter (Novinscak *et al*., 2009) as they are saprotrophs and can be pathogenic in a wide range of salinity

habitats. Some members of this taxon are parasitic (Chariton *et al*., 2015) and are abundant in soil. They were identified in abundance at the upper reaches of the estuary during sampling in 2019 (Figure 3.7, Table 3.1) which suggests a possible runoff from agricultural farms.

Environmental conditions, occurrences of algal blooms, and the amount and type of nutrients available significantly contributed to the structuring of bacterial population profiles in Sundays Estuary (Figure 3.10). With respect to bacterial communities, the most dominant bacterial groups throughout the water column at the time of sampling in 2018 and 2019 were Proteobacteria followed by Bacteroidetes, Actinobacteria, Firmicutes, and Cyanobacteria (Figure 3.11). While the same phyla were observed in a previous study of the Sundays Estuary in 2012, it was reported that Bacteroidetes dominated the water column (Matcher *et al*., 2018). This may be due to primer bias which is a well-known source of sequence variation in sequence data (Aird *et al*., 2011; Sabina & Leamon, 2015; Krehenwinkel *et al*., 2017; Elbrecht *et al*., 2018). However, the forward primer used in both this study and the one in 2012 (described in Matcher *et al*., 2018) were almost identical (with only a single base pair change from A to M) with only the reverse primer being different. While this would not completely alleviate the effect of any potential primer bias, any incurred biases would have been significantly mitigated. A more likely reason for the dominance of Bacteroidetes in 2012 is possibly a reflection of the changes in the nutrient inflows into the Sundays Estuary between 2012 and this current study (although no nutrient data was recorded in 2012). When comparing bacterial assemblages sampled a single year apart (i.e 2018 versus 2019) using the same methodologies, differences were observed albeit not to the same degree as that observed when comparing to results from 2012.

Within this current study (2018/2019), most bacterial phyla were widespread along the length of the estuary except Firmicutes and Cyanobacteria which were noticeably abundant at the head of the estuary in 2019. Since an estuary is formed when marine and freshwater meet, bacterial groups that were identified along the length of the estuary were a mixture of freshwater bacteria which were abundant at the riverine system and absent or decreased towards the mouth of the estuary and marine bacterial groups which were abundant at the mouth of the estuary and absent or decreased towards the head of

the estuary. This pattern was also reflected in studies by Fortunato *et al*. (2012) and Crump *et al*. (1999). Tidal forces pushed marine water farther into the estuary in 2019 resulting in a higher salinity gradient which likely increased the relative abundances of marine bacterial groups further up into the estuary. Salinity has a great impact on bacterial community composition and diversity in estuarine ecosystems (Urakawa *et al*., 2013; Zhang *et al*., 2014; Herlemann *et al*., 2016). This is indicated by the closer ordination of sites at the lower and middle reaches in 2019 (Figure 3.10B) with a high degree of positive correlation between salinity and the bacterial diversity observed.

The bacterioplankton in the upper reaches of the estuary exhibited a strong correlation with nutrient availability and was particularly evident in 2018 (Figure 3.10A). Not surprisingly, the co-occurrence of phytoplankton biomass also contributed to the bacterial population structure. DO, pH, and temperature are increased by high photosynthetic rate (Yang *et al*., 2015) hence their close association with bacterial structure at the middle reaches during algal blooms in 2018 (Figure 3.10A). The high growth of *Heterosigma akashiwo* can reshape bacterial community composition either by enhancing bacterial growth with their production of nutrients in the form of dissolved organic matter or by feeding on bacteria (Matcher *et al*., 2021). Some factors that have not been measured, such as heavy metals concentrations, grazing pressure, and levels of toxicity from the blooms, cannot be ruled out as they may have also contributed to bacterial richness and diversity but were beyond the scope of this study.

Phytoplankton contribute to global carbon fixation (Nelson *et al*., 1995), and most bacterial taxa that dominated the estuary were heterotrophs that use dissolved organic carbon as their source of energy. Although phytoplankton can be the primary source of carbon in a water source, bacterial richness at the upper reaches suggested that allochthonous organic carbon and other nutrient inputs were a more likely source of organic material than autochthonous inputs. The differences in bacterial community structure were evident even at the family level. Most of the bacterial groups that populated the bloom sites were degraders of organic matter. The second most abundant phylum, Bacteroidetes, which were in high relative abundance at the lower and middle reaches (Figure 3.11) are commonly associated with phytoplankton blooms (Buchan *et al*., 2014). *Flavobacteriaceae*, as the most dominant family in the Bacteroidetes phylum, were identified in high relative abundance at the lower and middle reaches while

Cryomorphaceae were in high relative abundance at the middle reaches in 2018 which may suggest a closer association with *Raphidophyceae* blooms. Most of the members of the *Flavobacteriaceae* family have been characterized as particle attached (D'Ambrosio *et al*., 2014) and are capable of degrading high molecular organic compounds such as the polysaccharides exuded by unicellular algae (Teeling *et al*., 2016) hence their abundance in the estuary.

Members of the *Cryomorphaceae* family, which is a sister group to *Flavobacteriaceae*, have also been identified as degraders of phytoplankton-derived organic matter (Teeling *et al*., 2016). The Bacteroidetes OTUs that were abundant at the bloom sites were mostly affiliated with *Cryomorphaceae*, however, some were affiliated with *Flavobacteriaceae* which belonged to the NS3a_marine_group genus. NS3a_marine_group are associated with algal blooms as well (Teeling *et al*., 2016), however, some of the NS3a_marine_group OTUs and that of *Cryomorphaceae* exhibited increased relative abundances at the upper reaches where there were high nutrient levels rather than high algal biomass. The most dominant Bacteroidetes OTUs at the nutrient-rich upper reaches had sequence identity to NS11-12_marine group. This bacterial taxon has a strong response to nitrates (Henson *et al*., 2018a) and it is not surprising that some NS11-12_marine group OTUs were found in higher relative abundances at the middle reaches in samples collected in 2019 than in 2018 because TOxN was higher at the middle reaches in 2019 than in 2018.

The phylum Proteobacteria consists of bacterial members with diverse metabolic potentials (Kersters *et al*., 2006), which is reflected in their widespread distribution throughout the Sundays Estuary. In this study *Burkholderiaceae* family was the most abundant at the head of the estuary, with OTUs with sequence similarity to *Hydrogenophaga*, MWH-UniP1_aquatic_group, Candidatus Fonsibacter ubiquis, and Candidatus_methylopumilus (Figure 3.12A). Members of this family are typically saprophytes and use organic matter as their source of carbon (Coenye, 2014), hence their high abundance at the upper reaches closest to the point of entry of the agricultural inputs and other plant organic matter particles. Site 8 was heavily inundated with reeds and other plant material, to the point where the sampling boat had difficulty maneuvering, which would have provided a rich source of plant material for degradation by the bacterial heterotrophs present in the water column.

Interestingly, Candidatus Fonsibacter ubiquis, (OTU65), which belongs to SAR11_clade, was present in significant numbers in the upper reaches of the estuary in 2019, despite members of the SAR11_clade being typically marine and one of the most abundant bacterial groups on the ocean surface (Steindler *et al*., 2011). Recently, the cultivation and genomic analysis of the first freshwater isolate belonging to SAR11_clade was reported (Salcher *et al*., 2011; Henson *et al*., 2018b). There is limited information on the response of Candidatus Fonsibacter ubiquis to agricultural influence in freshwater, however, Henson *et al*. (2018b) noted that freshwater SAR11_clade belongs to the subclade LD12, which are heterotrophs (Salcher *et al*., 2011). Though organic carbon was not measured in the water column, some bacterial species such as Candidatus methylopumilus utilize it as a source of energy, and this bacterial taxon can take up ammonium (Dennis *et al*., 2013; Salcher *et al*., 2015). OTU42, with sequence similarity to this bacterial species, was abundant at the upper reaches (Figure 3.12A). Moreover, Salcher *et al*. (2019) detected pathways in sulfur metabolism in members of the *Methylophilaceae* family. Although sulfur was also not measured in this study, it is a compound often found in chemicals used in agricultural farming including pesticides, insecticides, and fungicides (Gammon *et al*., 2010; Hinckley *et al*., 2020) as well as in manure and compost used in farms (Sager, 2012), therefore, it is likely that there was rainwater runoff from farming activities in the catchment area into the estuary. One noticeable difference in bacterial abundance between the two sampling periods was the presence of Clade_I with higher relative abundances at the lower and middle reaches in 2019 during the *Bacillariophyceae* blooms while it was not detected in 2018 (Figure 3.11). This taxon is marine species and has a close association with diatoms (Ortmann & Santos, 2016).

Proteobacteria OTU67 (99.5% sequence identity to Candidatus Pelagibacteria which is commonly found in marine environments) were in high relative abundance at the surface of the lower reaches in 2019 when the water had a high salinity gradient, however, it was also identified at 1 m and bottom of the water column at the upper reaches of the estuary. Interestingly, *Rheinheimera* (OTU6 and OTU20) was in high relative abundance during sampling in 2018 at the middle reaches of the estuary where there was a *Raphidophyceae* bloom as well as at the surface of the mouth of the estuary. Members of *Rheinheimera* have been reported to be of marine origin, however, it has since been isolated from different ecological niches including brackish water (Panda *et al.*, 2020). *Rheinheimera* showed anti-algal

activity in a study by Yang *et al*. (2015) and may play a role in the regulation of the algal blooms which occur regularly in the Sundays Estuary (Kotsedi *et al*., 2012).

Rhodobacteraceae decreased in relative abundance from the mouth to the middle of the estuary (Figure 3.11), however, OTUs of this family with sequence similarities to *Phaeobacter* and *Donghicola* were identified in high relative abundance at the middle of the estuary in 2018 during *Raphidophyceae* blooms (Figure 3.12A). *Rhodobacteriaceae* play a role in sulfur and carbon biogeochemical cycling (Pujalte *et al*., 2014) and are linked to phytoplankton-derived organic matter decomposition (Tada *et al*., 2011). The OTUs affiliated with *Pseudohongiella* and *Halieaceae* were identified at the middle reaches as well. Members of *Halieceae* bacteria have been reported to be associated with algal blooms (Choi *et al*., 2018; Matcher *et al.,* 2021), while *Pseudohongiella* belongs to the order *Oceanospirillales* which thrive well in an environment with high salinity levels and utilize organic compounds as their main source for metabolism (Aires *et al*., 2018; Sun *et al*., 2020). *Halieaceae* belongs to the order *Cellvibrionales* which are copiotrophs that prefer environments rich in detritus and have the potential to degrade polysaccharides (Spring *et al*., 2015). A higher fraction of members of the *Alteromonadaceae* were abundant at the lower reaches of the estuary and increased to the bottom of the water column correlating with an increase in chl-*a* concentration at the same sampling site in 2018 (Figure 3.11, Figure 3.3). *Alteromonadaceae* are capable of proliferating during algal blooms (Yang *et al*., 2015) and have been linked to high levels of organic carbon and can exploit a range of carbohydrates (Ivanova *et al*., 2004; Dinasquet *et al*., 2013; López-Pérez & Rodriguez-Valera, 2014). Moreover, members of this family can generate algicides during the late stages of algal blooms (Seyedsayamdost *et al*., 2011).

Sporichthyaceae and *Microbacteriaceae* were the only dominant Actinobacteria families that were identified in Sundays Estuary. They play a critical role in recycling nutrients such as the decomposition of organic materials like humus (Goodfellow & Williams, 1983). *Microbacteriaceae* were consistently abundant along the length of the Sundays Estuary in 2018 but decreased in relative abundance at the upper estuary in 2019, while *Sporichthyaceae* were in high relative abundance at the upper estuary in both years (Figure 3.11). This bacterial taxon is associated with compost (Normand, 2006) and high

nitrogen and phosphorus concentrations (Zhang *et al*., 2020b) and it is not surprising that it was found in high relative abundances at the upper reaches of the estuary closer to the farms. Candidatus_Planktophila sulfonica (OTU10) which belongs to the *Sporichthyaceae* family was in high relative abundance at the upper reaches of the estuary in both years. Candidatus Planktophila were first identified in freshwater (Hahn, 2009) as also shown in this study. They belong to the *acI* lineage which is auxotrophic. *AcI* lineages can get their carbohydrates and other carbon sources from terrestrial sources (Pérez & Sommaruga, 2006). Actinobacteria correlated negatively with nutrient resources in a study by Haukka *et al*. (2006) and the results of this study show no correlation between *Microbacteriaceae* and the nutrient levels nor observed phytoplankton blooms. Whilst *Microbacteriaceae* were constantly abundant along the length of the estuary in 2018 sampling (Figure 3.11), one of its dominant OTUs with high sequence identity to Candidatus Aquiluna (OTU27), was in high relative abundance at the lower and middle reaches of the estuary. Candidatus Aquiluna was isolated from eutrophic conditions since they can utilize phytoplankton-derived organic matter (Hahn, 2009), and were found at the algal bloom sites in this study. The dominant OTU with sequence similarity to Candidatus Limnoluna rubra (OTU58), was identified in higher relative abundances at the upper reaches of the estuary. This bacterial taxon also belongs to the *acI* lineage and is mostly identified in freshwater environments (Pérez & Sommaruga, 2006).

Cyanobacteria and Firmicutes were the least abundant phyla along the estuary and were only observed in significant relative abundances in 2019. Cyanobacteria grow best in freshwater with high nutrient concentrations (Kaselowski & Adams, 2013) which correlates well with its abundance at the upper reaches in 2019. *Nostocaceae* of the phylum Cyanobacteria can withstand harsh conditions (Berg & Sutula., 2015) and they thrived at sites 7 and 8 during sampling in 2019 (Figure 3.11). *Dichospermum-floss aquae* uses high levels of nitrates for growth (Kapkov *et al*.,2019) and OTU57 with sequence similarity to this bacterium was also in high relative abundance at the upper reaches (Figure 3.12A). *Erysipelotrichaceae* of the phylum Firmicutes were high in relative abundance in the upper reaches of the estuary in 2019 (Figure 3.11). Members of this family are capable of degrading organic matter and are associated with compost (Weglarz *et al*., 2018) hence their presence in high abundance at the upper reaches.

Sundays Estuary has shown to be a dynamic ecosystem containing a diverse planktonic community and high primary productivity. Agricultural inputs in this estuary are doing more damage than good which was evident with harmful algal blooms resulting from anthropogenic nutrients inputs, and an imbalance between phytoplankton and other planktonic community members. The eukaryotic community in the estuary was mostly autotrophic and the bacterial community was predominantly heterotrophic. Differences observed in the eukaryotic and bacterial community profiles between 2018 and 2019 were most likely due to dominance by different algal bloom species rather than changes in the annual anthropogenically-sourced nutrient input. The bloom of *H. akashiwo* in 2018 and *Cyclotella* in 2019 is a direct reflection of anthropogenic nutrient input into the estuary. Several of the other species of phytoplankton noted to be present in the Sundays Estuary have been reported to have the capability to become harmful algal blooms. Though the dataset generated was only for planktonic communities, it is clear that there is a possible negative impact on the food web. When looking at the bacterial structure in Sundays Estuary, it is evident that anthropogenic nutrients mapped their distribution patterns and composition. Although nutrients and occurrences of algal blooms impacted bacterial population profiles in the estuary, statistical analysis showed salinity to be the major driver of bacterial communities in the estuary. Many of the bacterial species observed in the estuary are known to be associated with the occurrence of harmful agal blooms and are likely to be involved in the degradation of phytoplankton-derived organic matter.

CHAPTER 4: SEASONAL VARIATION IN BACTERIOPLANKTON COMMUNITIES ALONG THE LENGTH OF THE URBANIZED/INDUSTRIALIZED IMPACTED SWARTKOPS ESTUARY

4.1 Physico-chemical characteristics of Swartkops Estuary

Swartkops Estuary exhibited a clear salinity gradient along its length which increased from the mouth to the upper estuary at the time of sampling in all seasons (Figure 4.1). The sites closer to the mouth of the estuary (sites 1 and 2) were influenced by the incoming marine water (> 30 ppt), while the rest of the estuary was mesohaline (5 – 18 ppt) and polyhaline (18 – 30 ppt) except at site 6 in the upper reaches which was classed as freshwater (< 5 ppt) at the time of sampling in autumn and winter. Higher salinity levels were observed at the upper reaches of the estuary during the spring season with readings ranging between 15.7 – 22.9 ppt. There was very little difference in the salinity gradient through the water column of site 1 from across all seasons, which indicates a high degree of mixing with a strong marine inflow. A typical salt wedge structure is clearly evident in the estuary with sites 2 to 5 showing strong vertical stratification which is more clearly delineated in autumn and winter and is most likely due to higher freshwater input from the catchment areas, as well as decreased mixing throughout the water column (Figure 4.1). Dissolved oxygen was relatively consistent along the estuary with the exception of a large increase in the upper water column in summer with the dissolved oxygen reaching levels of 22.9 mg/L (Figure 4.1).

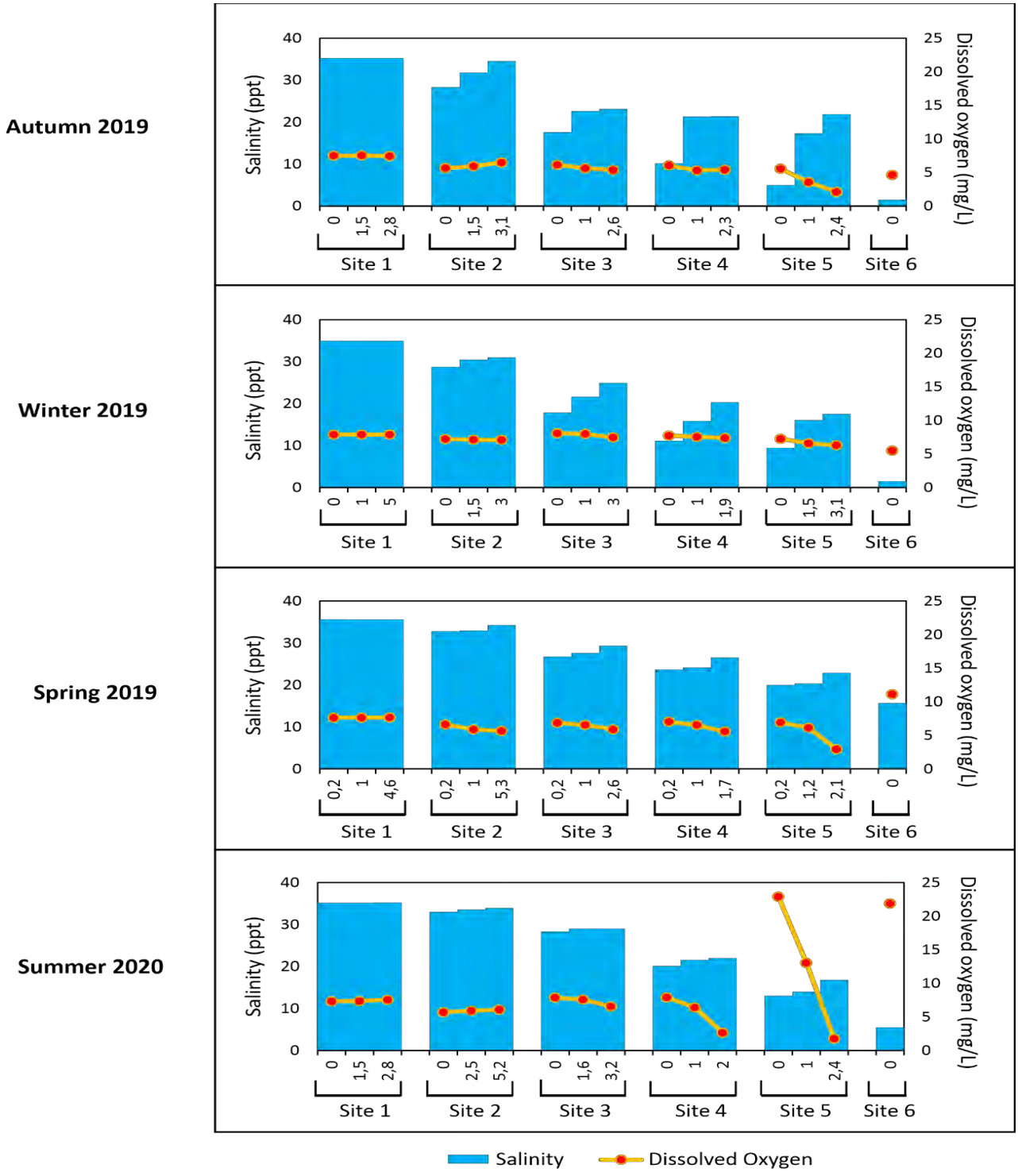

Figure 4.1: Seasonal variation in dissolved oxygen and salinity gradient through the water column (viz. surface, middle of the water column and bottom of the water column) along Swartkops Estuary. (Site 6 could not be accessed by boat and only surface water samples could be collected)

The temperatures varied with seasons with a maximum water temperature recorded as ~31°C in summer and coldest in winter with a minimum of ~13°C recorded at the time of sampling (Figure 4.2). The water surfaces recorded higher temperatures than the middle and bottom of the water column across seasons with a little to no difference between temperatures at the surface, middle and bottom of the water column at site 1. Temperatures through the water column did not vary greatly in winter (with the exception of site 4). During summer, and to a lesser extent during spring and autumn, water surface temperatures deviated from that of the rest of the column as one progressed up the estuary (sites 3-5) likely due to reduced mixing throughout the water column (Figure 4.2).

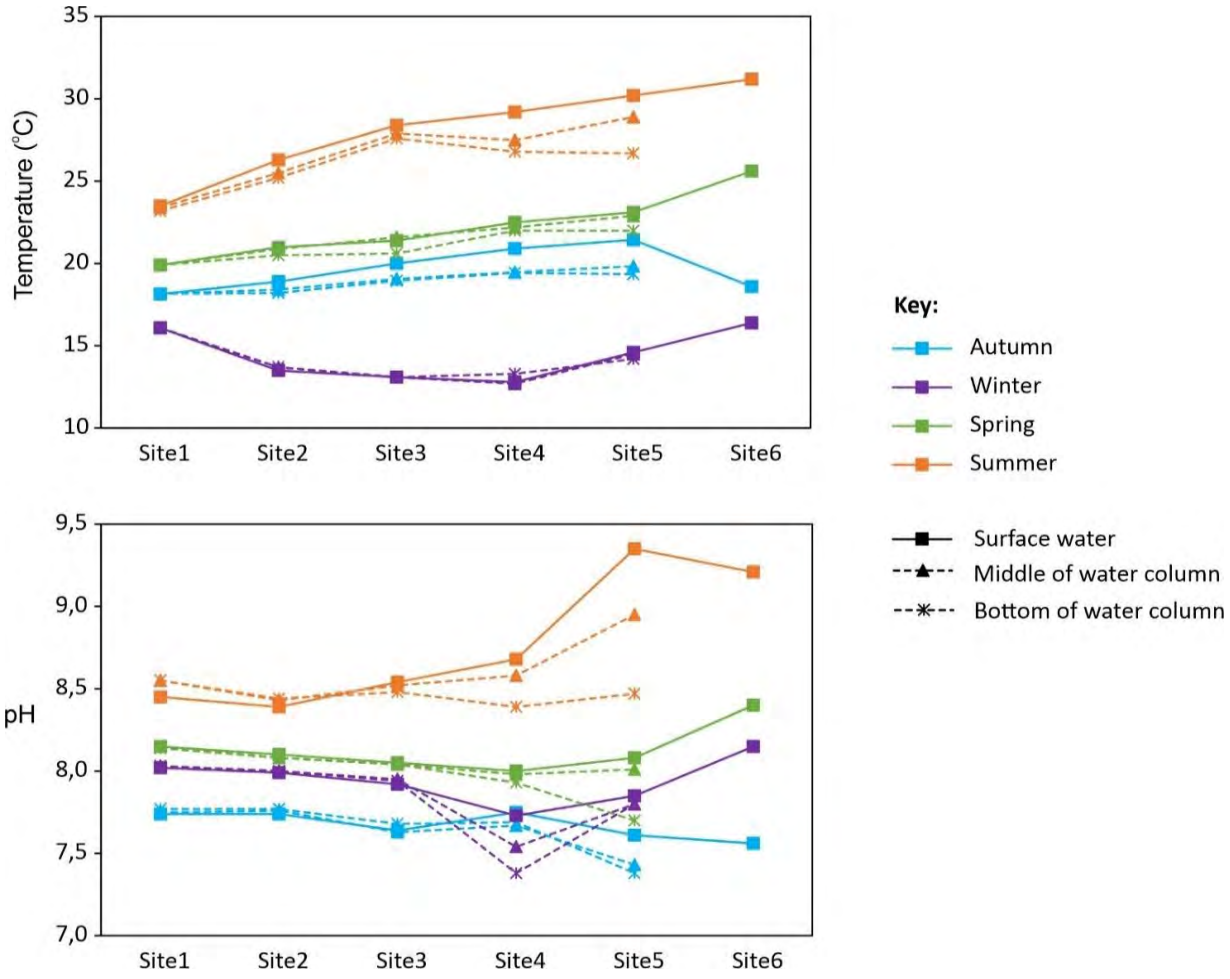

Figure 4.2: Seasonal variation in temperature and pH through the water column (viz. surface, middle of the water column and bottom of the water column) along the length of the Swartkops Estuary. (Site 6 could not be accessed by boat and only surface water samples could be collected).

The pH in the estuary was fairly consistent at the time of sampling in autumn, spring and winter varying between 7.38 and 8.4 (Figure 4.2). pH is inversely related to temperature (Bhusal, 2017) and increases with diatom blooms (Hinga, 1992). According to Figure 4.2, temperature had little effect on pH probably due other physical factors in the water which are beyond the scope of this research, while a higher pH was exhibited during diatom blooms (Figure 4.4) in the upper reaches at the time of sampling in summer, with a maximum pH of 9.35.

The concentrations of total oxidised nitrogen (TOxN), phosphate, ammonium and silicate recorded along the estuary varied seasonally (Figure 4.3). High nutrient levels were recorded at the upper reaches with a decrease towards the lower reaches.

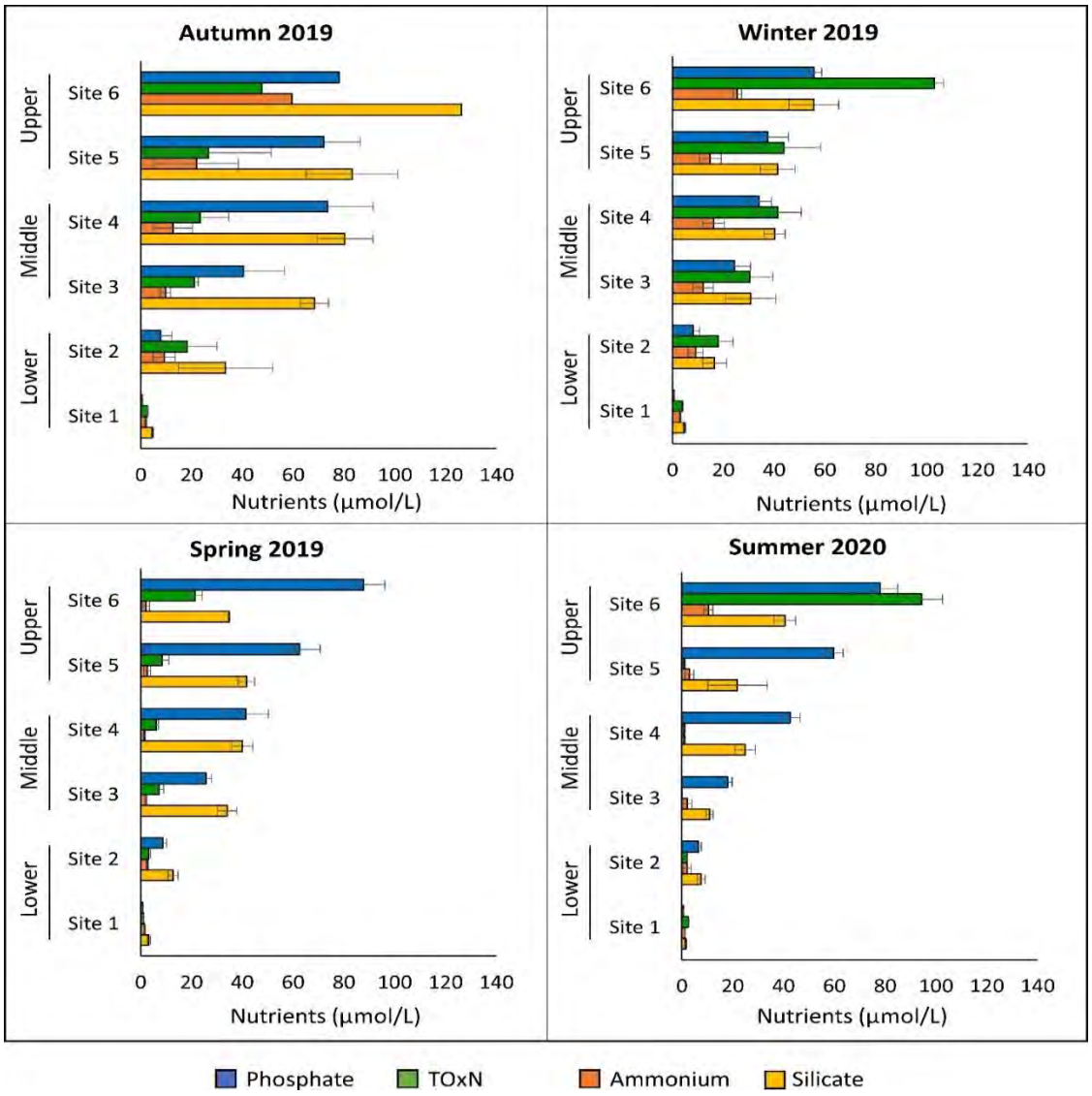

Figure 4.3: Average concentrations of total oxidised nitrogen (TOxN), phosphate, ammonium and silicate measured seasonally through the water column along Swartkops Estuary. (n = 9).

High concentrations of silicate and ammonium were observed during the autumn sampling with concentrations ranging between 4.58 – 126.2 µmol/L and 1.9 – 59.4 µmol/L respectively (Figure 4.3). Phosphate concentrations in the water column in the upper reaches of the estuary were consistently high irrespective of the season with concentrations at site 6 ranging from 55.8 µmol/L (winter) to 87.7 µmol/L (spring). TOxN concentrations were significantly higher at site 6 in winter and summer with values of 103.2 µmol/L and 94.3 µmol/L respectively. While much

lower concentrations of TOxN and ammonium were noted in spring, concentrations of phosphate (87.7 µmol/L) and silicate (34.8 µmol/L) remained high in the upper reaches (Figure 4.3).

The amount of nitrogen (in TOxN) in Swartkops Estuary indicates that this estuary was mesotrophic (≥ 100 but <1,000 µmol/L) for the most part (with the exception of samples collected in summer), whilst the lower reaches were typically observed as oligotrophic (< 100 µmol/L) according to the recommended levels in estuaries (Lemley *et al*., 2015). Estuaries with phosphorus levels > 100 µmol/L are classified as eutrophic (Lemley *et al*., 2015). With respect to the Swartkops Estuary, site 6 was eutrophic with phosphorus (in phosphates) concentrations ranging from 600 µmol/L (winter) to 900 µmol/L (spring).

4.2. Phytoplankton biomass

Phytoplankton biomass was determined indirectly by chl-*a* concentration and directly by phytoplankton cell counts from samples collected seasonally from the surface, middle and bottom of the water column along the length of the estuary (Figure 4.4).

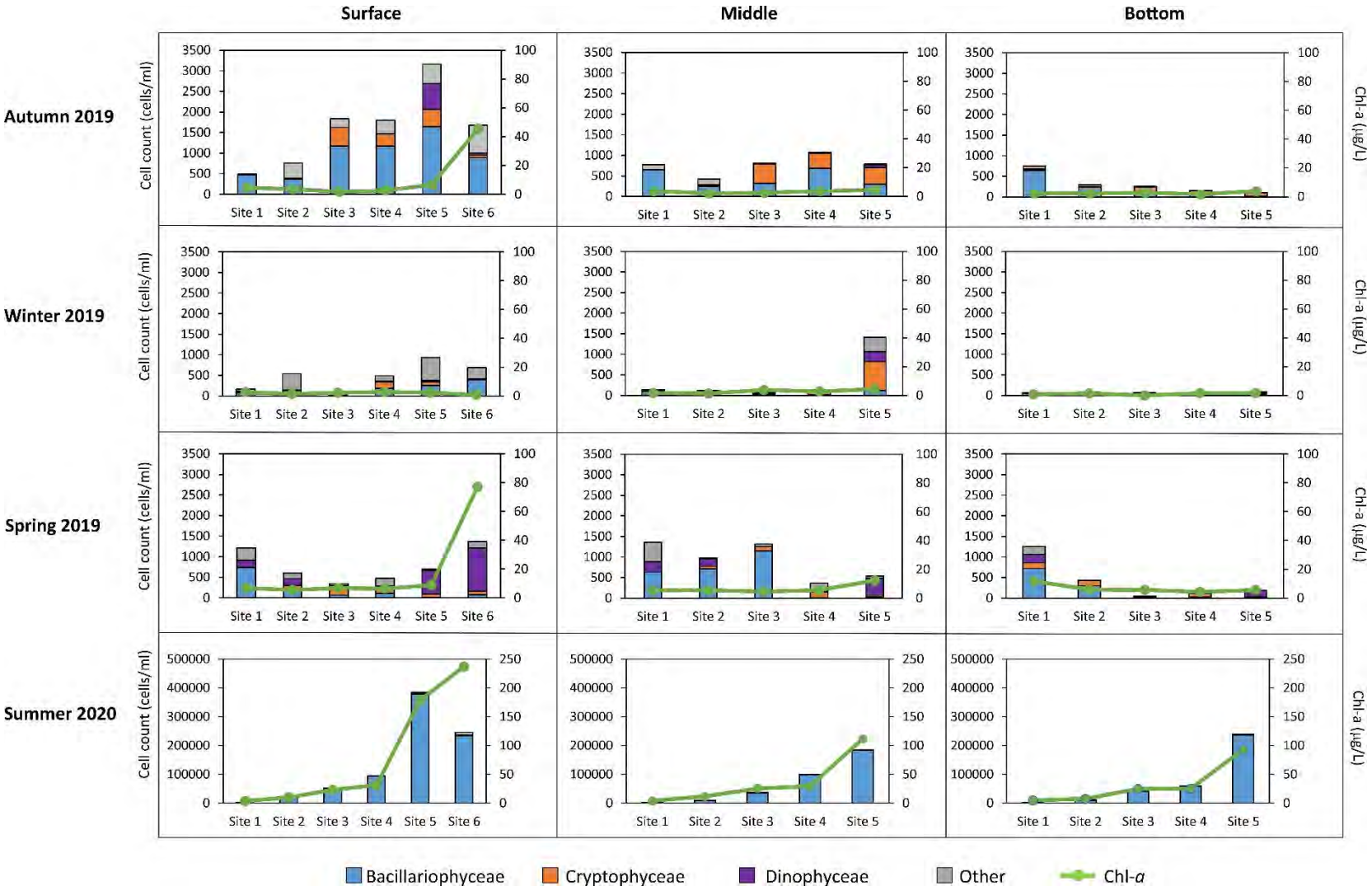

Figure 4.4: Phytoplankton biomass measured seasonally (2019/2020) through the water column along the length of Swartkops Estuary.

The Swartkops Estuary had high phytoplankton growth at the time of sampling in summer compared to samples collected from other seasons (Figure 4.4, note the values on the axes). The extremely high cell counts of *Bacillariophyceae* in summer are indicative of a bloom event (>20 chl-*a* µg/L) and likely the cause of lower nutrient concentrations (Figure 4.3) and increased dissolved oxygen content that was observed in the water column at site 5 in summer (Figure 4.1). The increase in chl-*a* concentration was also an indication of a bloom on the water surface of the upper reaches in autumn and spring sampling exercises. Overall, reduced abundances of phytoplankton was observed in samples collected in winter whilst intermediate levels of phytoplankton were noted in spring and autumn (Figure 4.4). *Bacillariophyceae* were widely distributed along the estuary during all sampling periods. *Dinophyceae* were more prevalent during spring and bloomed in the surface waters in the upper reaches of the estuary, whilst *Cryptophyceae* were more abundant in autumn and winter (Figure 4.4). Chl-*a* concentration did not always correlate with the cell count data, particularly in the surface water at the time of sampling in autumn and spring, as it is influenced by the phytoplankton cell sizes and concentration of chloroplasts which vary between phytoplankton species.

Of interest to note, *Raphidophyceae* were only identified during sampling in winter at very low abundances from the lower to the middle reaches while *Cyanophyceae* were abundant at the water surface of site 2 (Appendix 2; Figure S4). *Chlorophyceae* were abundant in the water surface, particularly in the upper estuary at the time of sampling in summer. Whilst the estuary had very low abundances of *Chlorodendrophyceae* during sampling in summer, they were abundant at the lower reaches of the estuary at the time of sampling in spring (Appendix 2; Figure S4).

4.3. Alpha Diversity

A total of 1 206 574 sequences remained in the dataset after curation and sub-sampling to even depth. Sequencing depth of samples collected seasonally from Swartkops Estuary was adequate to identify all the dominant bacterial OTUs as well as many of the less numerically prevalent

OTUs, as evidenced by the rarefaction curves nearing asymptote (Figure 4.5). Bacterial richness and diversity, as measured by Chao1 and InvSimpson, varied between seasons through the water column along the length of Swartkops Estuary (Figure 4.6). Whilst Chao1 richness index indicated that not all of the potential OTUs present in the ecosystems in this study were captured in the dataset, approximately 50 - 80% of all OTUs were represented in the dataset and the rarefaction curves confirm that all dominant OTUs were captured. This ratifies the suitability of the dataset as a good reflection of the bacterial community and a reliable representation of the majority of the diversity present.

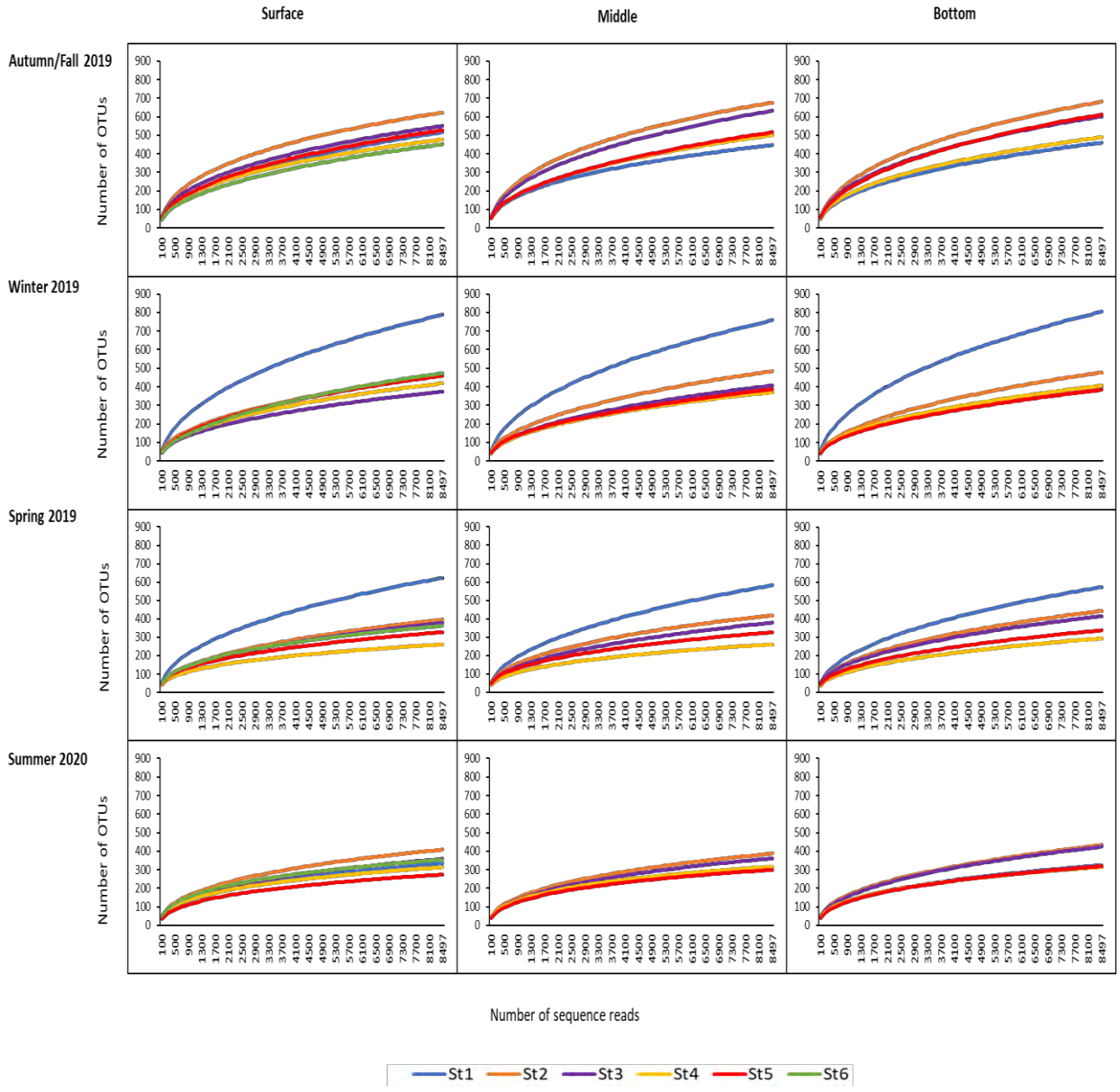

Figure 4.5: Rarefaction curves of bacterial OTUs (generated at distance of 0.03) collected seasonally (2019/2020) from the surface, middle and bottom of the water column along Swartkops Estuary. Subsampling was done at 8497 sequences.

Bacterial species richness varied seasonally along the length of Swartkops Estuary (Figure 4.6). An overall high bacterial richness was exhibited at the time of sampling in autum and winter whilst low richness was observed in summer during blooms (note the axes). The bottom of the water column at sites 2, 3 and 5 had higher richness than the surface and middle of the water column during sampling in summer, while a relatively consistent richness was observed from site 2 up the estuary at the time

of sampling in winter. Site 1 had the highest species richness during sampling in winter than in all other seasons. Bacterial species richness declined from the lower to the upper estuary at the time of sampling in the spring season.

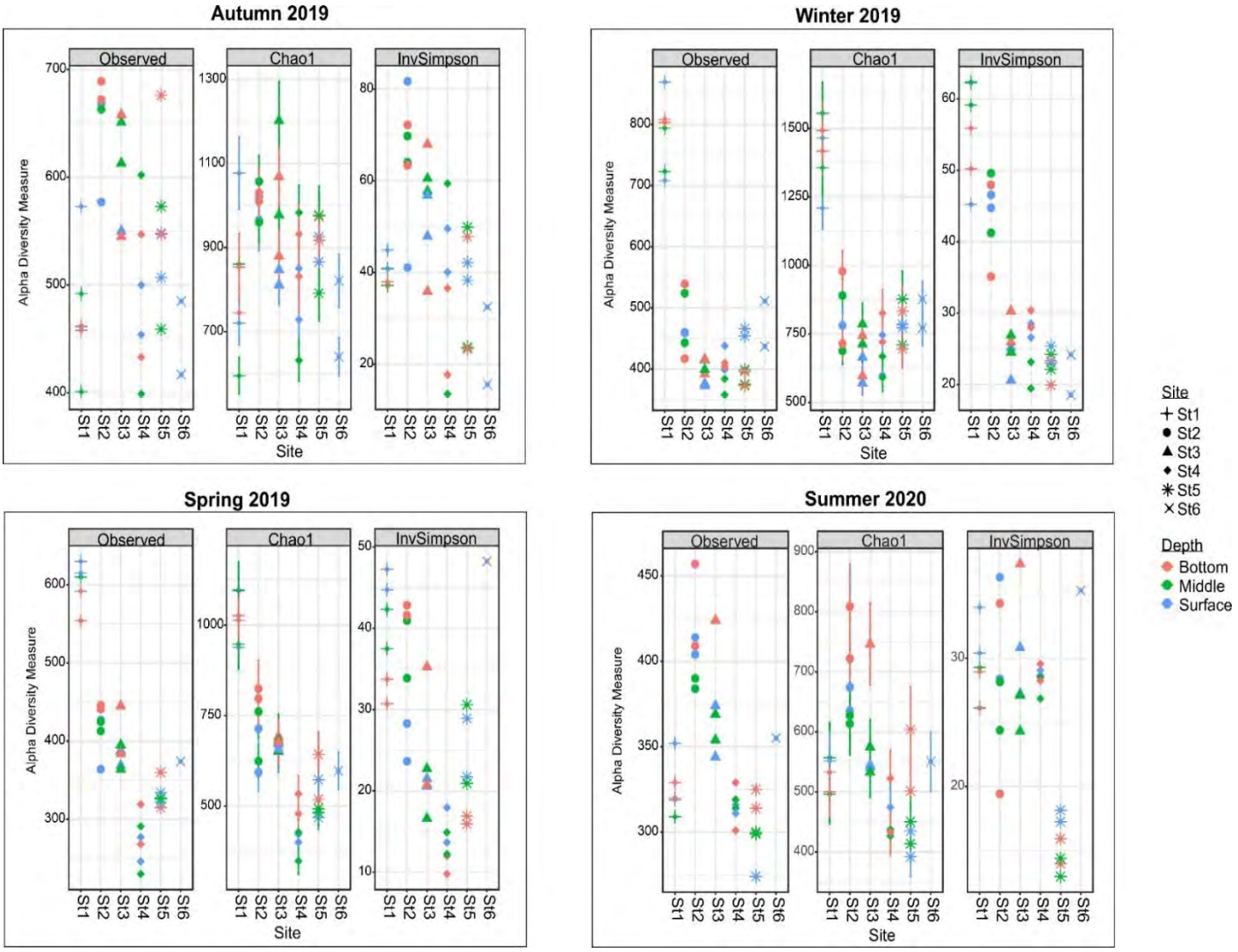

Figure 4.6: Bacterial species richness (Chao1) and bacterial richness and evenness (InvSimpson) through the water column along the length of Swartkops Estuary in all seasons throughout the year. (Site 6 could not be accessed by boat and only surface water samples could be collected).

Bacterial diversity, as evidenced by InvSimpson values (Figure 4.6), was low at the time of sampling in summer particularly at site 5 which coincided with the occurance of the *Bacillariophyceae* bloom (Figure 4.4). A decrease in diversity towards the upper reaches was observed during sampling in winter

and autumn seasons (Figure 4.6). Notably, samples collected from site 6 in spring and summer had higher bacterial diversity when the water was mesotrophic, and lower at the time of sampling in autumn and winter when the site was predominantly comprised of freshwater.

4.4. Beta Diversity

4.4.1. Principal Coordinate Analysis (PCoA) and Nonmetric multidimensional scaling (NMDS)

Different seasons had different environmental conditions which influenced the bacterial community structure in Swartkops Estuary. The NMDS and PCoA plotted in Figure 4.7 indicate the similarity and variation in bacterial community composition between seasons. The dataset was subjected to square root transformation so that the dominant OTUs will have comparible influence on variation in bacterial community composition between sites as that of the less dominant OTUs.

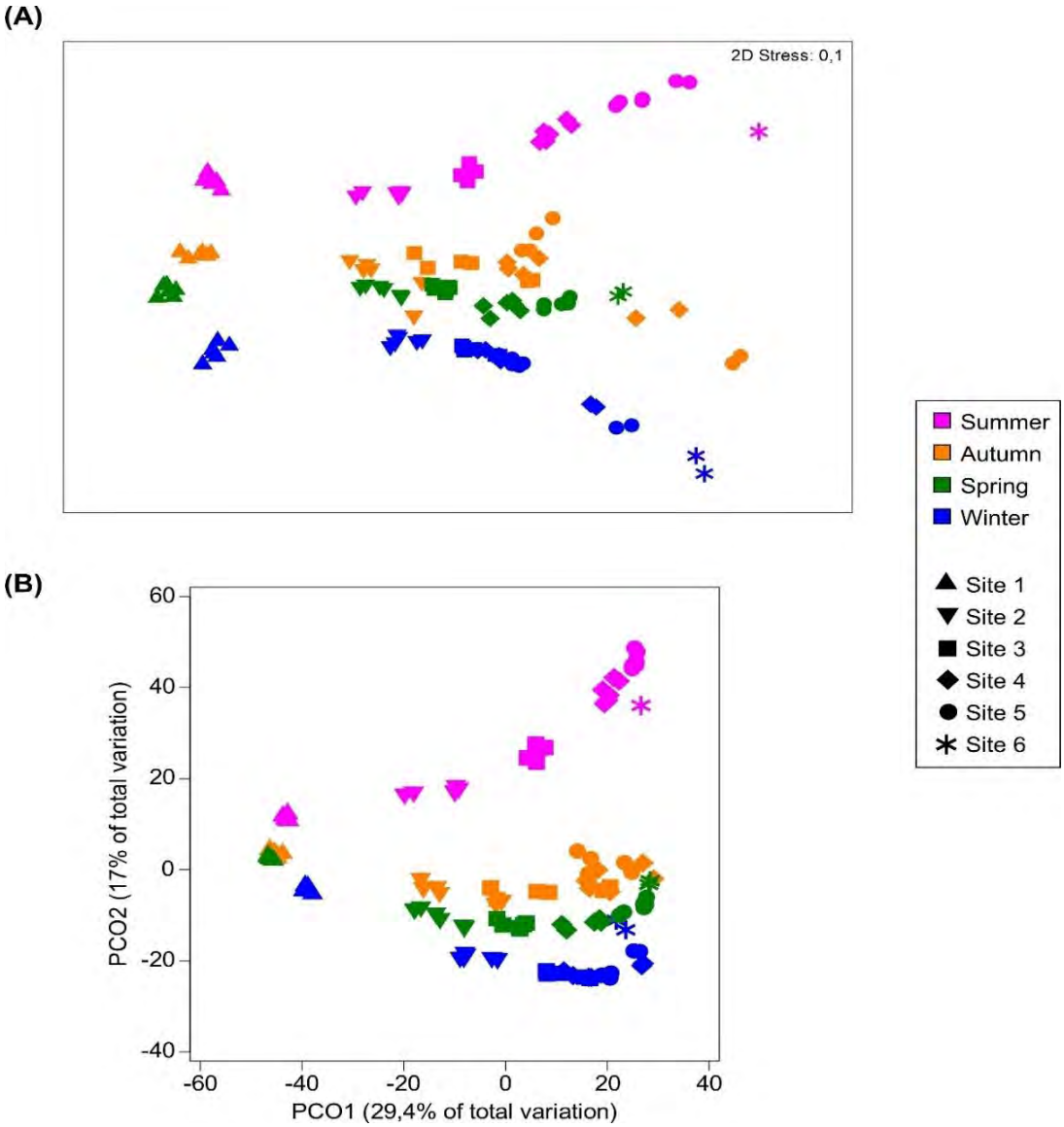

Figure 4.7: Bacterial OTUs variation analysis between seasons by NMDS (A) and PCoA (B). OTUs were generated at a distance of 0.03. The ordination analysis were run on Bray Curtis similarity derived from the square root transformed abundance.

There was an overlap of bacterial population profiles between seasons with a p-value of 0.001 and R-value of 0.376 (ANOSIM analysis) (Figure 4.7B, Appendix 2: Table S8). The degree of separation/difference between seasons was low (R-value < 0.5) except for the samples collected during winter and summer (p-value = 0.001, R-value = 0.571, Appendix 2: Table S8). This is also shown in the plots (Figure 4.7) where there was a noticeable separation in sampling sites

between summer and winter, while samples collected in spring and autumn showed greater overlap.

The different environmental variables experienced in each season were accountable for the observed variation along the length of the estuary (Figure 4.8). This is highlighted by statistical analysis (Spearman rank correlation) showing positive correlation between bacterial distribution patterns and environmental variables in samples collected from all seasons (Spearman rank correlation, Rho > 0.658, p = 0.001) (Appendix 2, Table S10). Swartkops Estuary was dominated by brackish water in the upper reaches through to marine water at the lower reaches and the statistical results (DistLM with AICc selection criteria) showed salinity as the major driver of variation between the sites, accounting for 41.02%, 48.6%, 57.66% and 62.04% of the total variation in the biological data in autumn, winter, spring and summer respectively (Appendix 2, Table S11). Sampling sites were classed based on salinity as oligohaline, mesohaline, polyhaline and marine and subjected to ANOSIM analysis. The variation between samples collected from oligohaline, mesohaline, polyhaline and marine sites in Swartkops Estuary was significant with p-value of 0.001 and R-value of 0.55 (winter) to 0.747 in summer (Appendix 2; Table S9).

Statistical analysis of correlation (BEST) highlighted all nutrients that were measured and dissolved oxygen as the best variables that correlated with distribution patterns in autumn, while salinity, temperature and silicate best correlated with distribution patterns in winter (Appendix 2, Table S10). Whilst salinity and silicate were highlighted as the best variables that correlated with distribution patterns in spring, temperature and phosphate were the best variables that correlated with bacterial distribution patterns in summer (Appendix 2, Table S10). Interestingly, silicate was one variable that best correlated with distribution patterns in all seasons except in summer, which may be due to exhaustion of this nutrient by diatoms. It is not surprising that chl-*a* which was a poor proxy for phytoplankton biomass (Figure 4.4) least explained the distribution patterns in samples collected across seasons (Appendix 2, Table S11).

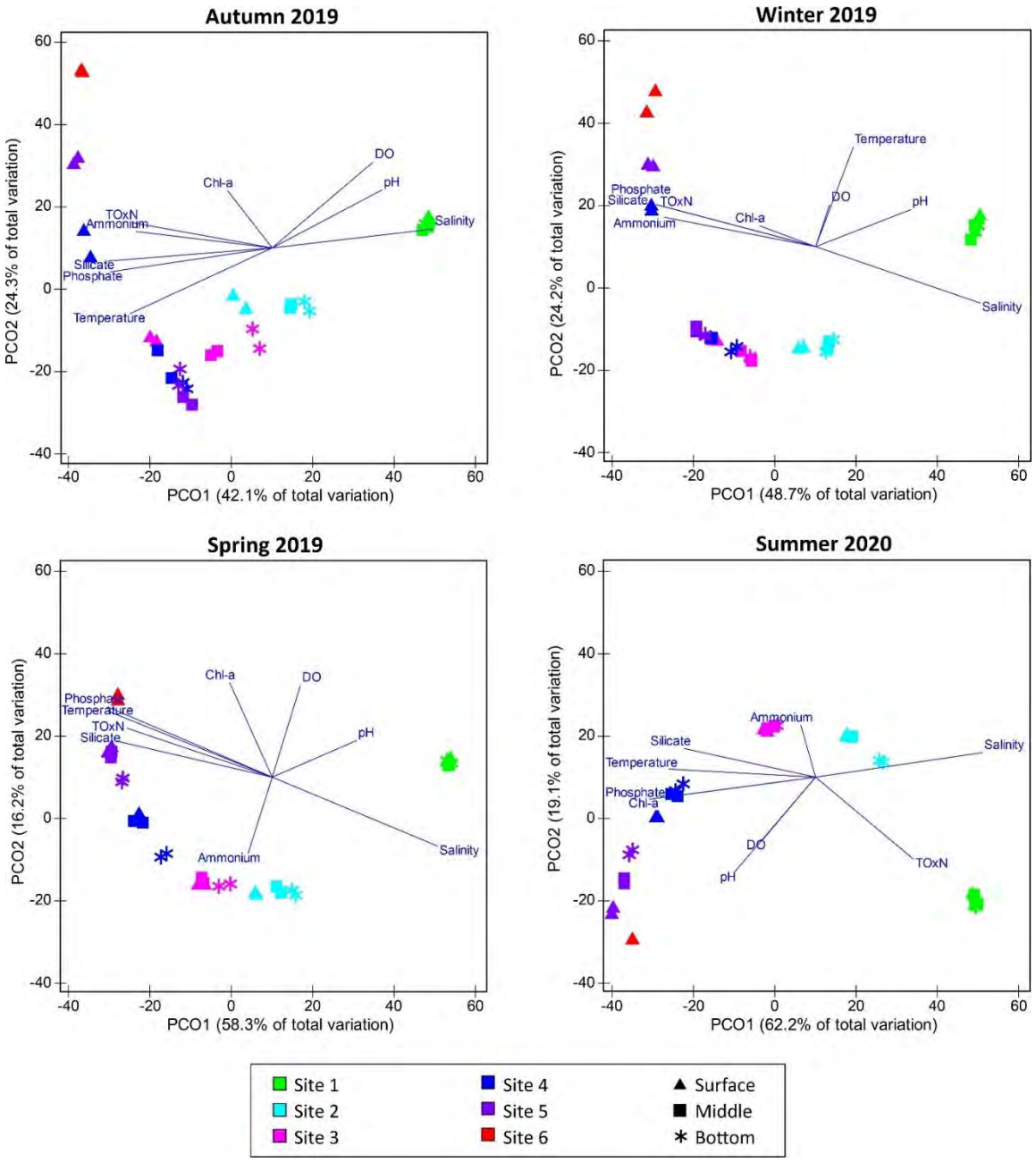

Figure 4.8: Principal Coordinate Analysis (PCoA) illustrating (dis)similarity between bacterial OTUs in samples collected seasonally at vertical and horizontal gradients through the estuary. Environmental variables driving bacterial OTUs compositional variation along the length of Swartkops Estuary are overlayed. PCoA was run on Bray Curtis similarity matrices derived from the square root transformed abundances of OTUs computed at a distance of 0.03.

4.4.2. Taxonomic classification

The differences between sampling sites that were displayed at OTU level (Figure 4.8) were also exhibited at the taxonomic level of family (Figure 4.9) with a shift in relative abundances of bacterial taxa along the length of the estuary. A clear difference can be observed in the surface waters for each of the seasonal sampling with distinctly different bacterial families dominating during different seasons. The surface waters had high relative abundance of *Rhodocyclaceae* and *Burkholderiaceae* which decreased downstream during sampling in autumn. *Burkholderiaceae* were also found in high relative abundance in the water surface at the upper reaches of the estuary during sampling in winter. Notably, *Cyanobiaceae, Rubritaleaceae* and the unclassified Proteobacteria were high in relative abundances through the water column in the upper reaches during sampling in summer, and decreased downstream while they were in very low relative abundance in other seasons. Samples collected at the middle and bottom of the water column in the upper reaches and water surface of site 3 during winter season, had high relative abundance of *Oxyphotobacteria* while members of this taxon were in low abundance during sampling in other seasons (Figure 4.9).

Alphaproteobacteria were consistently abundant in the middle and bottom of the water column along the length of the estuary during sampling throughout all seasons, except in summer where they decreased in relative abundance to the upper reaches. Some bacterial communities such as Clade_I (SAR11_clade), Clade_III (SAR11_clade), *Rhodobacteraceae* and *Flavobacteriaceae* were prevalent in all sampling seasons regardless of depth. Clade_I were present in low abundances at the time of sampling in summer while they were in high abundances in other sampling seasons particularly in spring. Whilst *Flavobacteriaceae* decreased in relative abundance from the lower to the upper reaches during sampling in summer, they were consistently abundant along the length of the estuary during sampling in other seasons. The lower reaches of the estuary with high salinity had high relative abundances of *Rhodobacteraceae* and *Cryomorphaceae* which decreased up the estuary. Members of *Cryomorphaceae* were noticeably high in relative abundance at the time of sampling in autumn particularly at the middle and bottom of the water

column of sites 2 and 3, while the water column at site 1, closer to the mouth of the estuary, had high relative abundance of SAR86_clade which were in low relative abundance during sampling at other sites of the estuary and in all other seasons.

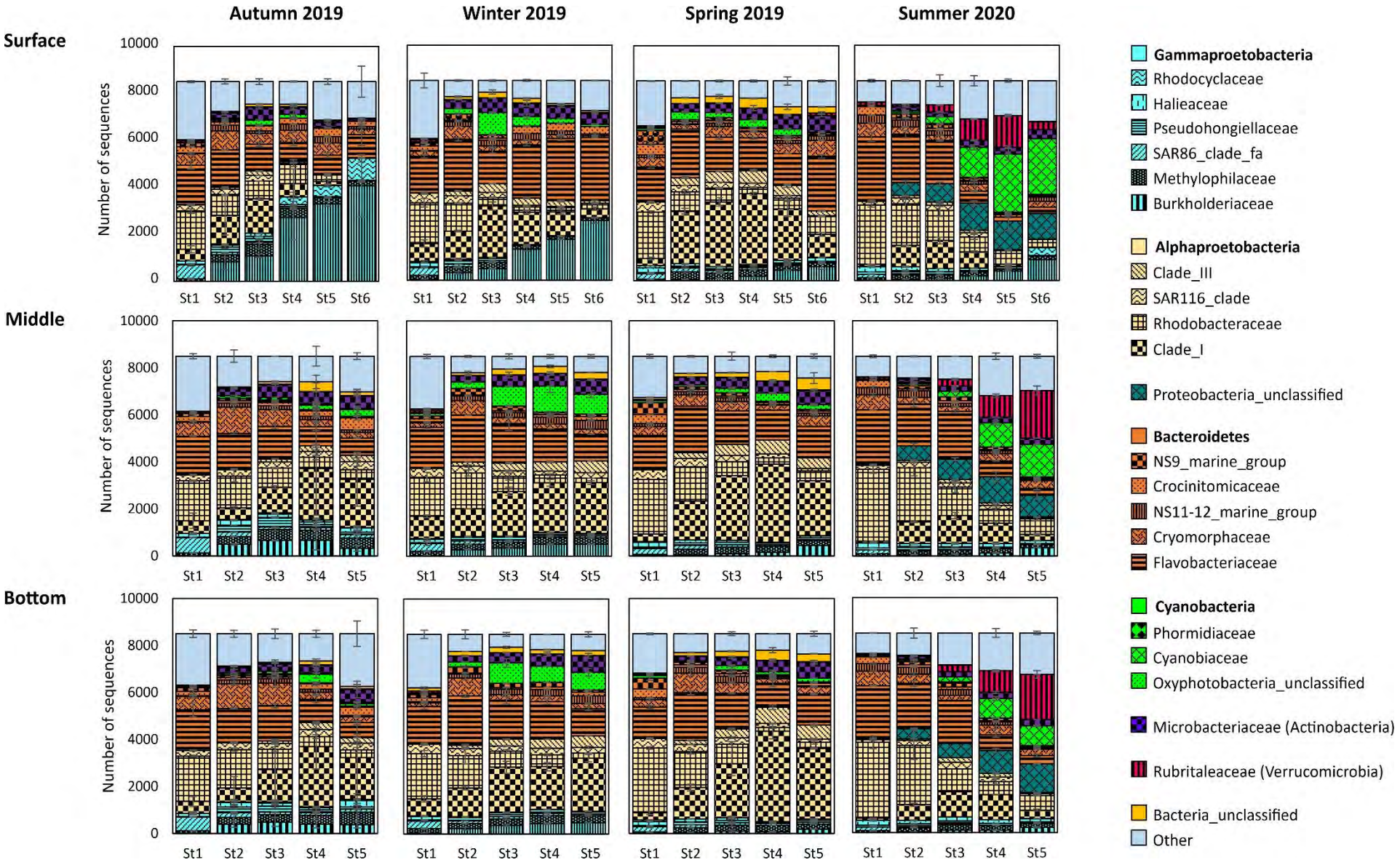

Figure 4.9: Taxonomic characterization of the bacterial communities at the taxon level of family in the surface, middle and bottom of the water column collected seasonally along Swartkops Estuary.

4.4.3. Dominant bacterial OTUs

Seasonal changes in the presence/absence and abundance of bacterial communities along the length of Swartkops Estuary were observed at the taxon level of family (Figure 4.9), down to taxonomic level of genus/species (Figure 4.10). The heatmaps provided in this chapter show the top 10 most dominant bacterial OTUs. The OTUs were aligned (BLAST) against bacterial sequences from the GenBank nr/nt database and GenBank RefSeq_rna sequences database (Appendix 2, Table S12). The OTUs of interest were highlighted in the heatmaps (Figure 4.10 A,B,C). Sequence similarities that were less than 97% were regarded as not significant while those that were >97% were significant.

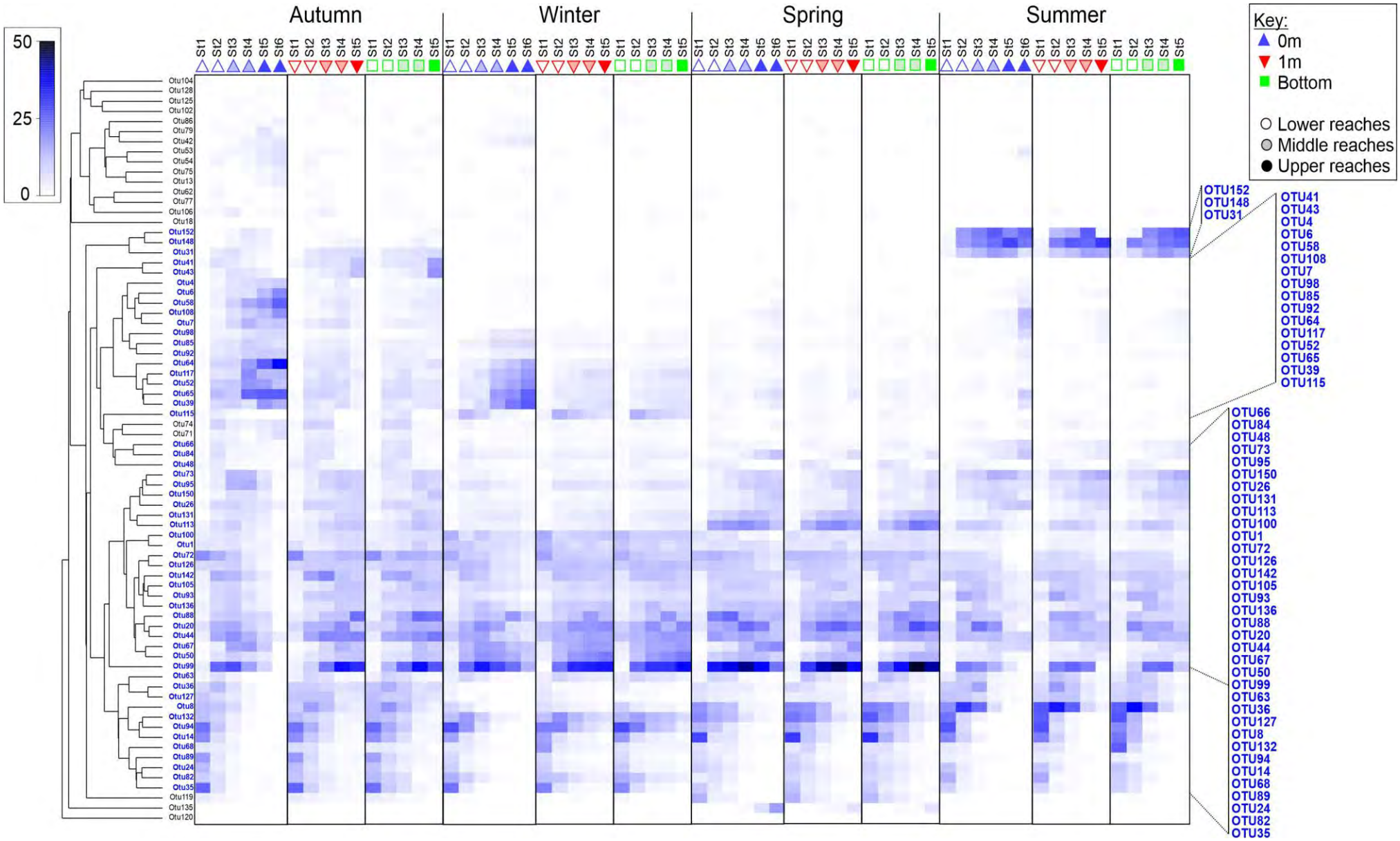

Figure 4.10A: Heatmap of the top 10 most numerically dominant OTUs of the phylum Proteobacteria sampled seasonally from the water surface, middle and bottom of the water column along the length of Swartkops Estuary. OTUs were clustered at distance of 0.03 and data was square root transformed. OTUs of interest are highlighted in blue color.

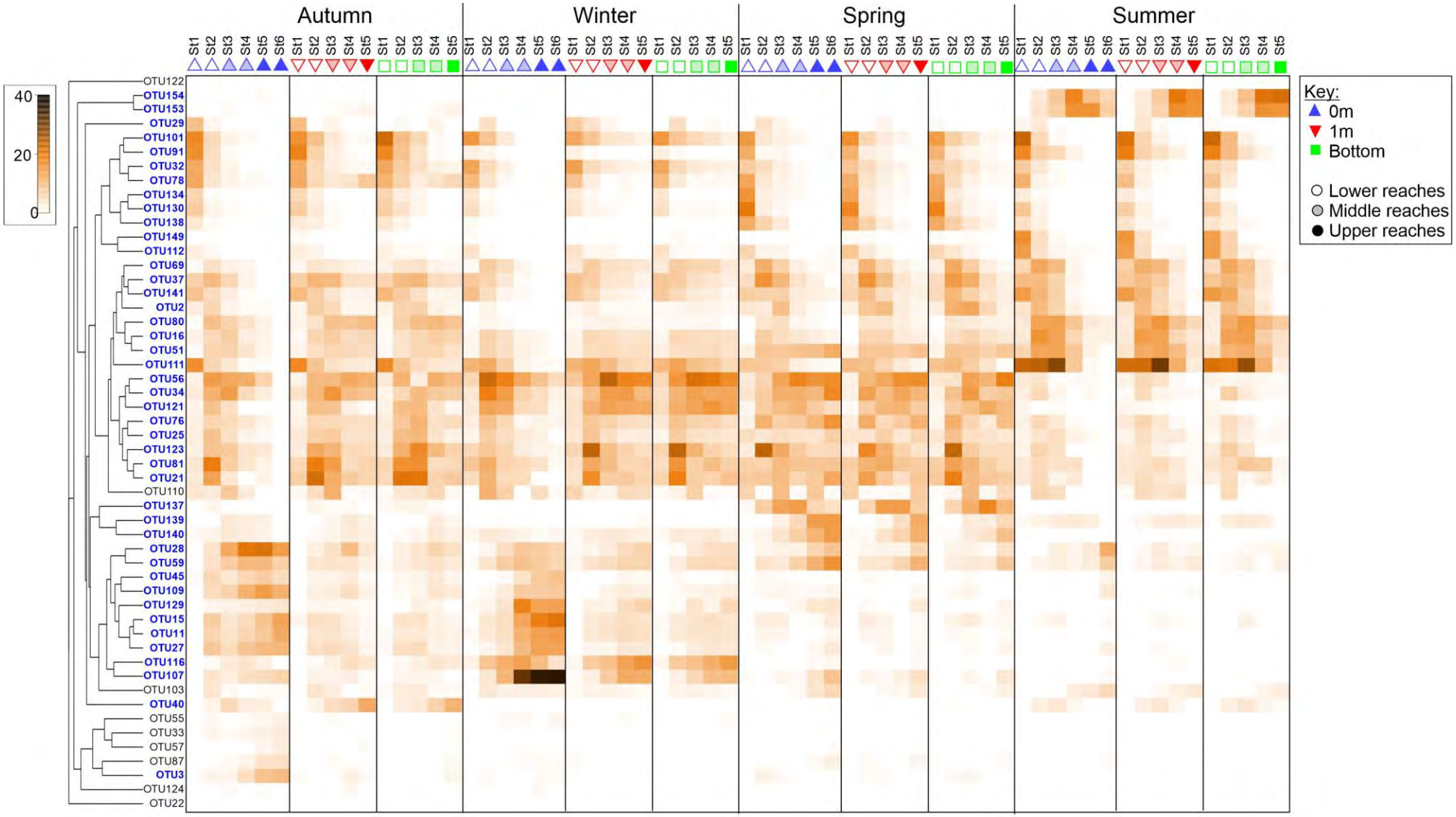

Figure 4.10B: Heatmap of the top 10 most dominant OTUs of the phylum Bacteroidetes collected seasonally from the water surface, middle and bottom of the water column along the length of Swartkops Estuary. OTUs were clustered at distance of 0.03 and data was transformed to square root. OTUs of interest are highlighted in blue color.

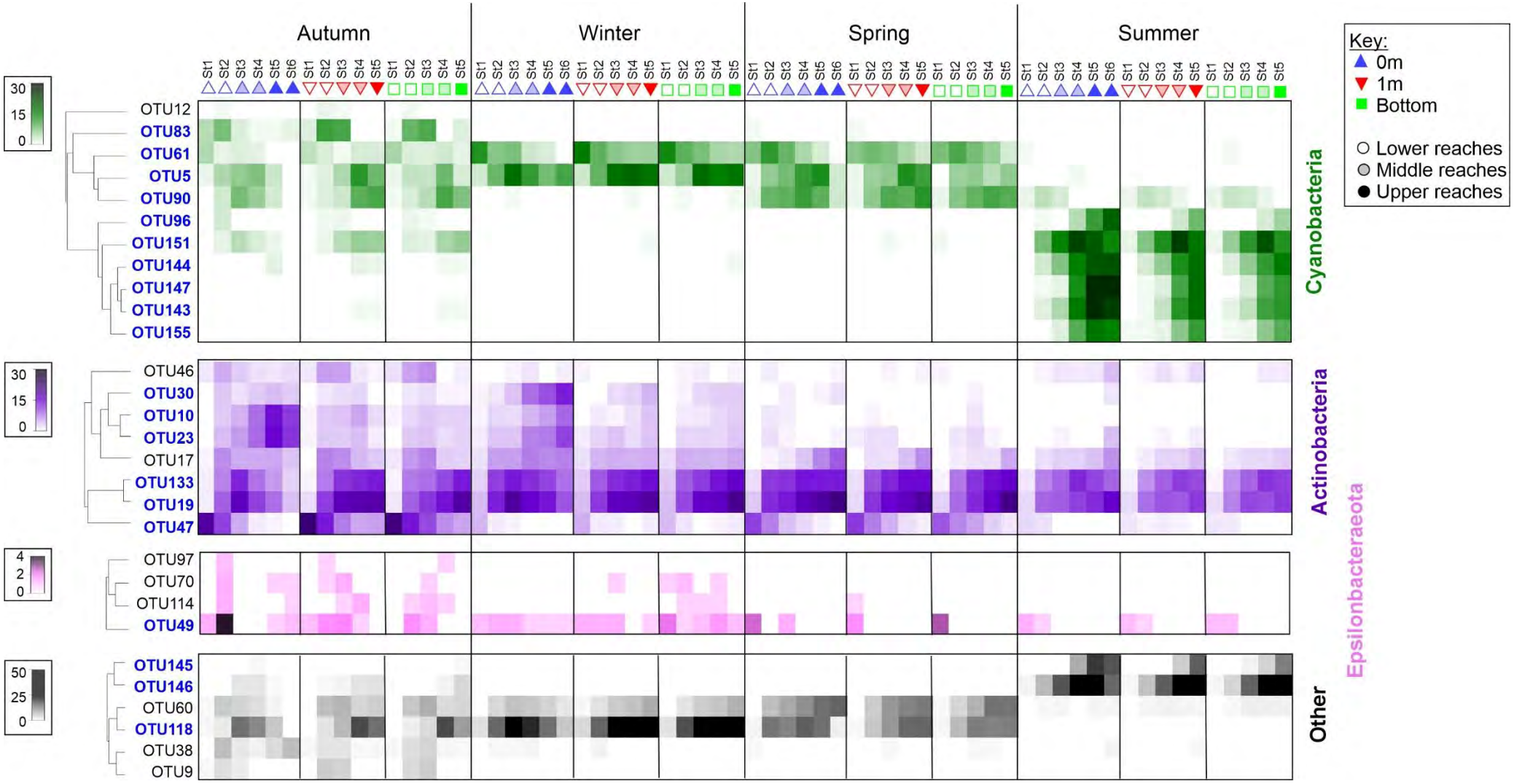

Figure 4.10C: Heatmap of the top 10 most dominant OTUs of the phyla Cyanobacteria, Actinobacteria, Epsilobacteraeota and other bacterial phyla (Verrucomicrobia, Planctomycetes, Tenericutes and Bacteria_Unclassified) column collected seasonally from the surface, middle and bottom of the water column along the length of Swartkops Estuary. OTUs were clustered at distance of 0.03 and data was transformed to square root. OTUs of interest are highlighted in blue color.

Most of the numerically dominant OTUs in Swartkops Estuary belonged to the phylum Proteobacteria. Notably, OTU6, OTU58, and OTU108 were numerically abundant in the water surface of the upper estuary during sampling in autumn, while they were relatively low in abundance in other sites of the estuary and in samples collected during other seasons (Figure 4.10A). OTU6 and OTU108 both showed high sequence similarity (>98%) to *Rhodocyclaceae* bacterium, C39*,* (Appendix 2, Table S12) which correlates with the results in Figure 4.9.. OTU58 showed 98% sequence similarity to *Limnohabitans australis.* OTU4 (98.78% sequence similarity to *Curvibacter* sp.), OTU85 (97% sequence similarity to *Methylobacillus glycogenes*), OTU92 (98.3% sequence similarity to *Hydrogenophaga defluvii*), as well as OTU98 and OTU7 which did not display significant sequence similarity with any genera in the reference database, were present in the water surface along the estuary and were absent/low in abundance at site 1 at the time of sampling in autumn. OTU108, OTU7, OTU98 as well as OTU65 (99% sequence similarity to *Limnohabitans planktonicus*), OTU39 (98.78% sequence similarity to *Polynucleobacter duraquae*), OTU84 (98.3% sequence similarity to *Idiomarina aestuarii*), and OTU66 were identified in the water surface of site 6 in summer in low relative abundance.

OTU152, OTU148 and OTU31 were noticeably abundant throughout the water column from site 2 to the upper reaches in summer and were found in low/absent numbers from samples taken during the remaining seasons. Unfortunately, these OTUs did not share any significant sequence similarity with any of the bacterial genera represented in the NCBI reference databases. Some OTUs with sequence similarity to *Limnohabitans* and *Polynucleobacter* species were found in high relative abundance during sampling in both autumn and winter (specifically OTUs 64, 117, 65 and 39) (Figure 4.10A, Appendix 2, Table S12). These bacterial OTUs decreased in relative abundance from the upper to the lower reaches and were found predominantly in the surface waters. As with the instances of OTU64, OTU65, OTU117 and OTU39, OTU52 (with 97.3% sequence similarity to *Hydrogenophaga taeniospiralis*, Appendix 2, Table S12) was also most frequently observed in the surface water datasets generated from the autumn and winter sampling efforts (Figure 4.10A).

OTUs showing sequence similarity to Candidatus Pelagibacter ubique (OTUs 20, 72, 88, 100, 1, 136 and 99; Appendix 2, Table S12) were identified along the length of the estuary and were in abundance at different sampling sites along the estuary in all seasons (Figure 4.10A). Of all the Proteobacteria OTUs, OTU99 was noticeably abundant through the water column of the middle to the upper reaches during all sampling periods, with a remarkably high relative abundance during the spring sampling in site 4. Some bacterial OTUs such as OTU132, OTU94, OTU14, OTU24, and OTU82 appeared to be in increased abundances primarily at the lower reaches of the estuary irrespective of the season during which sampling was conducted (Figure 4.10A). OTU8, OTU36 and OTU127, with sequence similarity to *Aliishimia ponticola* (Appendix 2,Table S12), were abundant at the lower reaches in autumn, spring and summer. OTU8 was the most plentiful through the water column from site 1 - 3 in spring and summer, while OTU68 was found in high relative abundance in the middle and bottom of site 1 during sampling in winter and summer respectively (Figure 4.10A). The lower reaches also had high relative abundance of OTU35 which was found through the water column of site 1 during sampling in autumn and winter, OTU119 which was in high relative abundance through the water column of site 1 in spring sampling, and OTU89 which did not share any sequence similarity with any genera in the NCBI reference database, which was numerically rich through the water column of site 1 during sampling in autumn.

Some OTUs were prevalent in the estuary from site 2 to the upper reaches irrespective of the season. These include OTU142 (98.5% Gammaproteobacteria bacteria), OTU44 (96.3% sequence similarity to *Methylotenera*), OTU67 (99.5% sequence similarity to *Hydrogenophaga*), OTU63 (96.35% sequence similarity to *Planktomarina*), and OTU126, OTU105, OTU50 and OTU93 which did not share significant sequence similarity with any genera in the reference database (Appendix 2, Table S12). These OTUs were absent/low in relative abundance in site 1. OTU113 showed increased relative abundances from site 2 to the upper reaches of the estuary during sampling in spring, although it was present in other seasons, it was numerically very low in abundance during winter sampling. The estuary had OTUs that were numerically abundant in all seasons except for winter and these included OTU73 (99.5% sequence similarity to *Ruegeria pomeroyi*), OTU150

(96.6% sequence similarity to *Parahaliea*), OTU95 (96.6% sequence similarity to *Donghicola*), and OTU26 and OTU131 which did not exhibit significant sequence similarity with any of the genera in reference databases (Appendix 2,Table S12). OTU115 (98.5% sequence similarity to *Lentibacter*) was distinctively numerous through the water column of sites 2 and 3 during sampling in winter, while OTU41 and OTU43 were plentiful at the middle and bottom of the upper reaches of the estuary during sampling in autumn and decreased down the lower reaches.

Within the Bacteroidetes phylum, OTU153 and OTU154 were noticeably prolific through the water column at the upper and middle reaches of the estuary during sampling in summer, while they were in low relative abundance during the remainder of the sampling seasons (Figure 4.10B). These OTUs showed no significant sequence similarity with any isolates within the reference databases used in this study (Appendix 2, Table S12). Another notable observation was the increased relative abundance of OTU111 in the summer dataset particularly through the water column of site 3, with a decrease towards the lower reaches of the estuary, although this OTU was present in the remaining seasons. OTU111 exhibited 99.27% sequence identity to *Formosa* sp. (Appendix 2, Table S12). OTU107, with 97.54% sequence identity to *Flavobacterium* sp. (Appendix 2, Table S12), was most abundant in the water surface of the upper reaches during sampling in winter and was also present in the middle and bottom of the water column and decreased downstream (Figure 4.10B). OTU15, OTU11, and OTU27 which also displayed sequence similarity to *Flavobacterium* sp., OTU45 (98.8% sequence similarity *Aquirufa* sp.), and OTU109 were also numerically abundant in the water surface of the upper reaches during sampling in winter as well as in the autumn sampling, and decreased downstream while they were low in abundance in other seasons. OTU129 was also in increased abundances on the water surface of the upper reaches during sampling in winter while it was low in abundance in other seasons. The upper reaches of the estuary had increased abundances of OTU28 and OTU59 which did not show significant sequence similarity with any genera in the reference database. These OTUs decreased in abundance down the estuary, however, OTU28 was in high relative abundance in the water surface at sites 4 and 5 during autumn sampling. OTU116, which showed no significant sequence similarity with genera in the reference database, was in high relative

abundance through the water column in the upper reaches in winter and decreased downstream. At the time of sampling in spring, OTUs 139 and 140 (98.8% sequence similarity to *Polaribacter pacificus*) were numerically abundant through the water column in the upper reaches and decreased downstream and were low in number in samples collected in other seasons, while OTU137 was numerically abundant through the water column in the middle reaches. OTU40 which did not share sequence similarity with any genera in the reference databases was plentiful in the middle and bottom of the water column at the upper reaches during sampling in autumn and decreased down the estuary while OTU3 was only abundant at the water surface (Figure 4.10B, Appendix 2, Table S12).

The lower reaches had a significant number of numerically dominant Bacteroidetes OTUs across seasons which include OTUs 29 and 101 with sequence similarity to *Tenacibaculum*, OTU91 (99% sequence similarity to *Flavobacterium* sp.), OTU130 (97% sequence similarity to *Olleya*), OTU149 (99% sequence similarity to *Polaribacter atrinae*) and OTUs 32, 78, 134, 138, 112, 69, 37, and 141 which did not exhibit significant sequence similarity with any genera in the NCBI reference database (Figure 4.10B, Appendix 2, Table S12). These OTUs decreased in relative abundances as one progressed up the estuary. Only OTU101 and OTU78 were consistently present throughout all sampling seasons, while OTU91 was numerically low in abundance through the water column at the time of sampling in winter, and OTU32 was low in abundance during sampling in spring and summer. Whilst OTU37 was in high relative abundance through the water column in sites 2 and 3 during sampling in spring and autumn, OTU134, OTU130, OTU138 were in high abundance particularly during sampling in spring, and OTU149 and OTU112 were numerically abundant during sampling in summer (Appendix 2, Table S12).

OTU80 (98.7% sequence similarity to *Flavobacterium* sp.) and OTUs 16 and 51, which did not have any closest sequence identity in the reference databases, were present through the water column from site 2 to the upper reaches and were numerically abundant at sites 2 and 3 in summer (Figure 4.10B, Appendix 2, Table S12). OTU80 was in very low numbers during sampling

in winter, while OTU2, with no significant sequence identity in the reference database, was plentiful at sites 2 and 3 in all seasons and was poorly represented in winter. Some Bacteroidetes OTUs were prevalent through the water column along the length of the estuary at the time of sampling in autumn, winter and spring, at varying degrees of occurance. These include OTUs 121 (98.5% sequence similarity to *Polaribacter* sp.), and OTUs 56, 34, 76, 25, 123, 81, and 21 which did not share significant sequence similarity with any genera in the NCBI reference database (Appendix 2,Table S12). All these mentioned OTUs were identified in low relative numbers during sampling in summer. OTU123 was prevalent through the water column in site 2 in spring sampling and middle and bottom of the water column in winter. OTU81 and OTU21 were in high relative abundance through the water column at site 2 during sampling in winter, and water surface of site 2 and middle-bottom of the water column at sites 2 and 3 during sampling in autumn (Figure 4.10B, Appendix 2, Table S12).

Of all the dominant Cyanobacteria OTUs that were identified in the water column of the estuary, OTU96, OTU151, OTU144, OTU147, OTU143 and OTU155 were noticeably prevalent at the upper reaches during sampling in summer, while they were numerically absent/low in abundance during sampling in other seasons (Figure 4.10C). OTU143, OTU144, OTU155 and OTU96 exhibited >97% sequence similarity to *Cyanobium gracile PCC 6307* (Appndix 2, Table S12), while OTU147 showed 97.8% sequence similarity to *Synechococcus rubescens.* Whilst OTU90, which could not be accurately classified against the reference database, ocurred in high relative frequency through the water column from site 2 to the upper reaches in autumn and spring sampling, OTU5 (98.8% sequence similarity to *Ostreococcus tauri*) was noticeably abundant through the water column from site 2 to the upper reaches during sampling in all seasons, with exception of samples collected in summer. OTU61, with 98.8% sequence similarity to *Ostreococcus lucimarinus,* was high in relative abundance through the water column in the lower to middle reaches during sampling in winter and spring, while OTU83 (98.3% sequence similarity to *Arthrospira*) was present in increased numbers at sites 2 and 3 during sampling in autumn, and was absent/low in abundance from the datasets generated for the remainder of the seasons.

Within the Actinobacteria, OTU133 (>98% sequence similarity to *Pontimonas*) and OTU19 (97% sequence similarity to Candidatus Limnoluna rubra) were consistently prevalent from site 2 to the upper reaches of the estuary across all seasons (Figure 4.10C, Appendix 2, Table S12). OTU47 was high in relative abundance at the lower reaches during sampling in autumn and spring, and decreased in relative abundance up the estuary, while it was in low relative abundance in samples collected from other seasons. OTU10 and OTU23 which were high in relative abundance in the water surface of the upper reaches at the time of sampling in autumn and winter and decreased towards the lower reaches, exhibited poor sequence identity with reference sequences in the database (Appendix 2, Table S12). OTU30 was prevalent in the upper reaches in autumn and winter (Figure 4.10C) but could not be accurately classified against the reference database.

OTU49 was the only OTU of the phylum Epsilonbacteraeota which was numerically prevalent at the water surface of site 2 in autumn (Figure 4.10C) and was present in the estuary during other seasons. BLAST analysis returned a 99% sequence similarity to *Arcobacter cibarius* (Appendix 2, Table S12). OTU145 and OTU146, with no significant sequence similarity in the NCBI reference database, were observed in high relative abundances through the water column in the upper to middle reaches in summer (Figure 4.10C, appendix 2, Table S12). In contrast, OTU118 was poorly represented in the summer sampling but was observed in winter and to a lesser extent in spring and autumn.

4.5. Discussion

The Swartkops Estuary is severely impacted by urban and industrial activities. The lower reaches of the Swartkops Estuary are highly populated, the middle reaches are closer to industries (e.g. brickworks, tanneries, wool industries, railway yards, etc.), while poorly managed/maintained wastewater treatment plants situated at the upper reaches, namely the KwaNobuhle, Kevin Jones and Despatch wastewater treatments plants, have been identified as the major sources of anthropogenic nutrients in Swartkops Estuary (Adams *et al*., 2019). This is consistent with the results of this study which showed mesotrophic to eutrophic phosphorus conditions and

mesotrophic nitrogen conditions for the most part of the estuary with the exception of samples collected in summer which showed oligotrophic conditions at some parts of the upper reaches (Figure 4.3). High densities of hyacinth plant growth was observed at the upper estuary and diatom blooms occurred during sampling in summer, at the upper reaches in autumn, and *Dinophyceae* bloomed at site 6 in the spring season (Figure 4.4). All of which is a clear reflection of the high anthropogenic nutrient levels within this impacted estuary. Diatoms need silicate and nitrogen for growth and there was a major bloom in summer which means that the concentrations of silicate and nitrogen entering into the estuary at this time was likely high (Figure 4.3). Silicon is essential for cell wall development of diatoms while nitrogen is needed for the production of molecules such as sugars, amino acids and nucleic acids (Martin-Jézéquel *et al*., 2000; Geider & La Roche, 2002; Yamada *et al*., 2014).

The physico-chemical variables and the amount and type of nutrients influence both bacterial and phytoplankton growth and metabolism. Furthermore, phytoplankton blooms themselves also affect bacterial composition and diversity (Peterson & Grimm, 1992; Luo *et al*., 2013; Sison-Mangus *et al*., 2016; Li *et al*., 2017). Previous studies have highlighted salinity as one of the major drivers in bacterial distribution patterns in estuarine ecosystems (Lozupone & Knight, 2007; Fortunato *et al*., 2012; Campbell & Kirchman, 2013), which corresponds to the results of this study where a strong correlation between salinity and bacterial community composition was observed (Appendix 2: Table S11). A peak in dissolved oxygen concentration during summer sampling correlated with algal blooms which may have resulted in the lower bacterial diversity in the estuary (Figure 4.6). The lowest species richness and bacterial diversity were observed at the time of sampling in summer during diatom blooms particularly at site 5 which correlated with the highest densities of diatoms (Figure 4.4, and Figure 4.6). Some phytoplankton produce exudates that can inhibit bacterial growth and limit diversity (Sison-Mangus *et al*., 2016; Shibl *et al*., 2020), and the blooms mostly supports a smaller and more specialised bacterial population that can degrade phytoplankton biomass and exudates hence the low species richness and diversity observed.

Most bacterial communities were prevalent along the estuary throughout sampling seasons but varied in abundance and distribution patterns (Figure 4.9). Though the estuary had lower species richness and bacterial diversity during sampling in summer (Figure 4.6), some bacterial taxa such as members of *Cyanobiaceae, Rubritaleaeceae* and the unclassified Proteobacteria were in high relative abundance at the upper estuary in bloom sites while they were identified in low relative abundance during sampling in other seasons (Figure 4.9). The phylum Verrucomicrobia*,* which includes *Rubritaleaceae,* are chemoorganotrophs which have diverse enzymes that degrade complex organic matter and algal polysaccharides (Hedlund, 2010; Martinez-Garcia *et al*., 2012; Cardman *et al*., 2014; Nitin Parulekar *et al*., 2017; Kopprio *et al.*, 2020) hence their presence in high relative abundances in sites with high *Bacillariophyceae* growth. They have also been found in activated sludge (Liu *et al.*, 2005) and it is not surprising that they were identified in high relative abundance at the upper reaches closer to sewage treatment plants. This taxon is capable of surviving under low nitrogen content as they have *nifH* gene which helps in nitrogen fixation (Yao *et al.,* 2014; Wang *et al*., 2016) which matches their high abundances in summer when nitrogen levels were lower. Cyanobacteria grow best in nutrient rich environments (Anbalagan & Sivakami, 2019) and under high illumination and warmer temperatures (Andreeva *et al.*, 2020) which are mostly experienced during summer which favoured the growth of *Cyanobiaceae* observed in this study (Figure 4.9). In contrast, *Oxyphotobacteria* were found in high relative abundance from samples collected during lower temperatures in winter (Figure 4.9). The upper reaches of the estuary had an abundance of numerically dominant OTUs which had sequence similarity to *Cyanobium* during sampling in summer which correlates with abundances of *Cyanobiaceae* and a few with *Synechoccus* identity. *Cyanobium* and *Synecchocus* produce toxins which can be a health risk (Genuário *et al*., 2018) to the surrounding communities.

The phylum Proteobacteria contains highly metabolically diverse bacterial species and was the most dominant phylum present in the Swartkops Estuary (Figure 4.9 and Figure 4.10A). The Alphaproteobacteria were the dominant subphylum observed in the middle and the bottom of the water column in all seasons with significantly high abundances of Clade_I (SAR11_Clade) and *Rhodobacteriaceae*. The SAR11_clade is the most abundant bacterioplankton in the oceans

(reviewed in Giovannoni, 2017) and has been reported in many estuarine ecosystems (Kan *et al.,* 2008; Campbell & Kirchman, 2013; Korlević *et al*., 2016). Within this taxon, Clade_I and Clade_III of the Candidatus Pelagibacteria were in high relative abundance along the length of the estuary particularly at the time of sampling in spring when salinity was higher than in other seasons (Figure 4.9). Most of the OTUs with sequence similarity to Candidatus pelagibacter ubique were also found along the length of the estuary in all seasons (Figure 4.10A) which is not surprising as Swartkops Estuary is marine dominated and provides a favorable environment for growth of these bacteria.

Rhodobacteraceae such as *Aliishimia* and *Amylibacter* have previously been isolated from marine environments (Nedashkovskaya *et al*., 2016; Kim *et al*., 2019) which corresponds to the abundance of OTU8, OTU36 and OTU94 at the lower reaches (Figure 4.10A). They have a close association with organic particles and compounds produced by phytoplankton growth (Campbell & Kirchman, 2013; Schveitzer *et al.*, 2020) hence the high abundance of OTU8 during the phytoplankton bloom in summer. *Sulfitobacter faviae,* which is a member of the *Rhodobacteraceae,* have previously been isolated from the marine environment (Kwak *et al*., 2014) and OTU14 with sequence identity to this bacterium was numerically abundant at the lower reaches throughout all seasons. Members of *Sulfiltobacter* have known algicidal activity (Barak-Gavish *et al*., 2018) and they are involved in sulfur cycling (Ivanova *et al*., 2004). Sulphur was not measured in this study and there are no reports specifically on sulphur concentrations in Swartkops Estuary, however, Olisah *et al.* (2019) reported high levels of chemicals that contain sulfur in the Swartkops Estuary.

In contrast to representation by Alphaproteobacteria, the surface waters in autumn exhibited a high relative abundace of Gammaproteobacteria and in particular members belonging to the family *Burkholderiaceae*. *Burkholderiaceae* are known for their saprophytic and denitrification ability (Ishii *et al*., 2009). A less striking but still significant relative abundance of this *Burkholderiaceae* was also observed at the water surface of the upper estuary in winter, while

the remainder of the sampling seasons had limited representation of this taxon in their respective datasets. Members of the *Burkholderiaceae* include *Polynucleobacter* (OTU39, OTU117) (Appendix 2, Table S12, Figure 4.10A) and *Limnohabitans* species (OTU58, OTU64, OTU65), including *Limnohabitans australis, Limnohabitans curvus* and *Limnohabitans planktonicus. Limnohabitans* are the recently identified bacteria that are mostly abundant in freshwater (Salcher *et al*., 2008; Hahn *et al*., 2010; Newton *et al*., 2011) and this is likely why OTUs with significant identity to these bacteria were not present in the marine dominated sites in the estuary. *Limnohabitans* have shown high growth on algal derived substrates and algal exudates and are reported to be prone to grazing by protists, however, their high growth potential compensates the high grazing losses (Jezbera *et al*., 2005; Šimek *et al*., 2010; Kasalický *et al.*, 2013; Salcher & Šimek, 2016). *Polynucleobacter* have genes associated with degradation of pesticides (Fang *et al.*, 2014) and since the upper reaches receives water from urbanised areas, there may have been contamination from pesticides used in lawns and from the limited agricultural activities in the catchment. The presence of *Polynucleobacter duraqueae* in high relative abundance at the upper reaches which had high levels of phosphorus is not surprising as they are chemoorganotrophic and are closely associated with humic compounds and phosphorus (Jezberová *et al*., 2010; Hahn *et al*., 2016; Shabarova *et al.*, 2017). *Hydrogenophaga* (OTU52) (family *Burkholderiaceae)* was found to be dominant in water surface at the upper reaches of the estuary in winter and autumn. This bacteria has the ability to degrade organic pollutants (Lambo & Patel, 2006) which were previously reported in high levels in Swartkops Estuary (Nel *et al.*,2015; Olisah *et al*., 2019). The water inflow may have deposited organic pollutants from the catchment into the water surface, since a higher amount of rainfall (ranging between 19 – 38mm) was recorded just prior sampling, as opposed to when sampling in spring and summer was conducted.

Bacteroidetes were the second most abundant phylum along the length of the estuary with high relative abundances of *Flavobacteriaceae* (Figure 4.9, Figure 4.10B). Members of *Flavobacteriaceae* degrade polysaccharides in water sources (Teeling *et al*., 2016; Gavriilidou *et al*., 2020). *Formosa arctica,* which was recently isolated from the arctic ocean (Kwon *et al*., 2014), and OTU111 with 99.27% sequence similarity to this bacterium was identified in high relative

abundance in brackish water at the middle reaches in summer as well as the lower reaches during sampling in autumn while it was identified in low relative abundance at the time of sampling in other seasons (Figure 4.10B). Members of *Formosa* are associated with high levels of organic matter (Mann *et al*., 2013). The organic matter was not measured in this study, but since there was high diatom growth in summer, there could be some diatom material being washed downstream as well as other organic matter from the Motherwell canal (see Chapter 5) whose entry point is close to site 3 in the estuary. Members of *Cryomorphaceae,* which are also known as particle attached bacteria that degrade polysaccharides, were found in all seasons along the entire length of the estuary with a slight increase in relative abundance during sampling in autumn (Figure 4.9).

Actinobacteria are commonly found in low salinity environments (Camarena-Gómez *et al.,* 2018) and can be associated with diatom blooms (Dziallas & Grossart, 2011; Bagatini *et al*., 2014), but they were low in relative abundance during diatom blooms in summer while they were in relative large proportions in other seasons. They play a role in decomposition and nutrient cycling (Zhang *et a*l., 2019). *Pontimonas* was previously isolated from coastal marine environment (Cho *et al*., 2018) and OTU133 with sequence similarity to this genus was identified along the length of the marine dominated Swartkops Estuary. Candidatus Limnoluna rubra was previously isolated from freshwater (Hahn, 2009) but OTU19 was present in brackish environments (Figure 4.10C). Planctomycetes and Epsilonbacteraeota were not amongst the most dominant phyla, nonetheless, OTU145 belonging to phylum Planctomycetes was identified in high relative abundance at the upper estuary during sampling in summer (Figure 4.10C). *Planctomycetes* species consume semilabile dissolved organic matter from the Cyanobacteria blooms (Kopprio *et al.*, 2020), even though there were no Cyanobacteria blooms at the time of sampling, there was high relative abundance of *Cyanobiaceae* at the upper estuary during sampling in summer. The presence of OTU49 with sequence similarity to *Arcobacter cibarius* (phylum Epsilonbacteraeota) in the estuary was an indication of sewage pollution from the highly populated lower reaches. *Arcobacter* species have previously been isolated from faecally contaminated environmental water sources (Collado *et al*., 2010; Hozumi *et al*., 2018).

Pollution in the estuary was also evidenced by high abundances of bacterial taxa that are associated with sewage waste (Figure 4.10 A,B,C). *Rhodocyclaceae* are mostly used as indicators of pollution and have been reported to be abundant in urban pollution (Newton & McLellan, 2015). They are known for their denitrification and pollutant degrading ability in wastewater treatment plants (Loy *et al*., 2005; Weisssbrodt *et al*., 2011) and were in abundance at the water surface of the upper reaches in autumn (Figure 4.9; Figure 4.10A). The genus C39 of the *Rhodocyclaceae* was previously isolated in abundance from storm flow in urbanised streams (Chaudhary *et al*., 2018). The last sampling site (site 6) at the upper reaches receives water from the Swartkops river which is frequently affected by the inflow from the poor maintained sewage treatment plants situated upstream (Kevin Jones, KwaNobuhle WWTP) (Adams *et al*., 2019).

Although Swartkops Estuary was marine dominated throughout all sampling season, which explains high abundances of marine bacteria in the estuary, urbanization and environmental variables defined its bacterial composition and diversity. This was evident with the presence of pathogenic bacteria and bacterial communities that are associated with sewage and increased nutrient levels in the estuary as well as algal blooms. Different environmental conditions were experienced in each of the seasons, which also had effect on bacterial community structure.

CHAPTER 5: THE IMPACTS OF ANTHROPOGENIC POLLUTANTS DISCHARGED BY WATER CHANNELS ON THE BACTERIOPLANKTON POPULATION STRUCTURE IN SWARTKOPS ESTUARY

5.1 Anthropogenic activities contributing to pollution in the water channels discharging into the Swartkops Estuary

Water inputs in Swartkops Estuary occur primarily from stormwater runoff and wastewater (treated or otherwise) from treatment plants and industrial activities along the length of the estuary. Much of this poor water quality enters Swartkops Estuary via canals (Motherwell canal, Markman canal), rivers (Chatty river and Swartkops river), and creeks (Tippers creek) (Figure 5.1). The Chatty river and Tippers creek run through a heavily urbanized area and discharge water into the lower reaches of the estuary. The Motherwell and Markman canals were constructed to discharge stormwater to the middle reaches of the estuary while the upper reaches receive water from the Swartkops river.

Tippers creek runs close to busy highways and residential areas, including Amsterdamhoek, where runoff from roads and stormwater carries pollutants into the creek. Since Tippers creek discharges closer to the mouth of the estuary, pollutants are likely to be flushed out of the estuary at a higher rate by tidal events than that of sites further up the estuary. The Chatty river passes through populated informal settlements (Missionvale, Redhouse, Kleinskool) before reaching the urbanized area where there are industries in the catchment and manholes at different places which frequently overflow into the river and eventually into the estuary. The surrounding communities use the water from the river for domestic purposes such as washing and irrigation and there are reports of people using the river as a dumping site for animal carcasses (Nel *et al.,* 2015). The industrial sites at proximity to the Chatty river at the lower reaches of the estuary include carbon black production plant, railway yards, and wastewater treatment works which may pollute the river and estuary through runoff or discharged effluents.

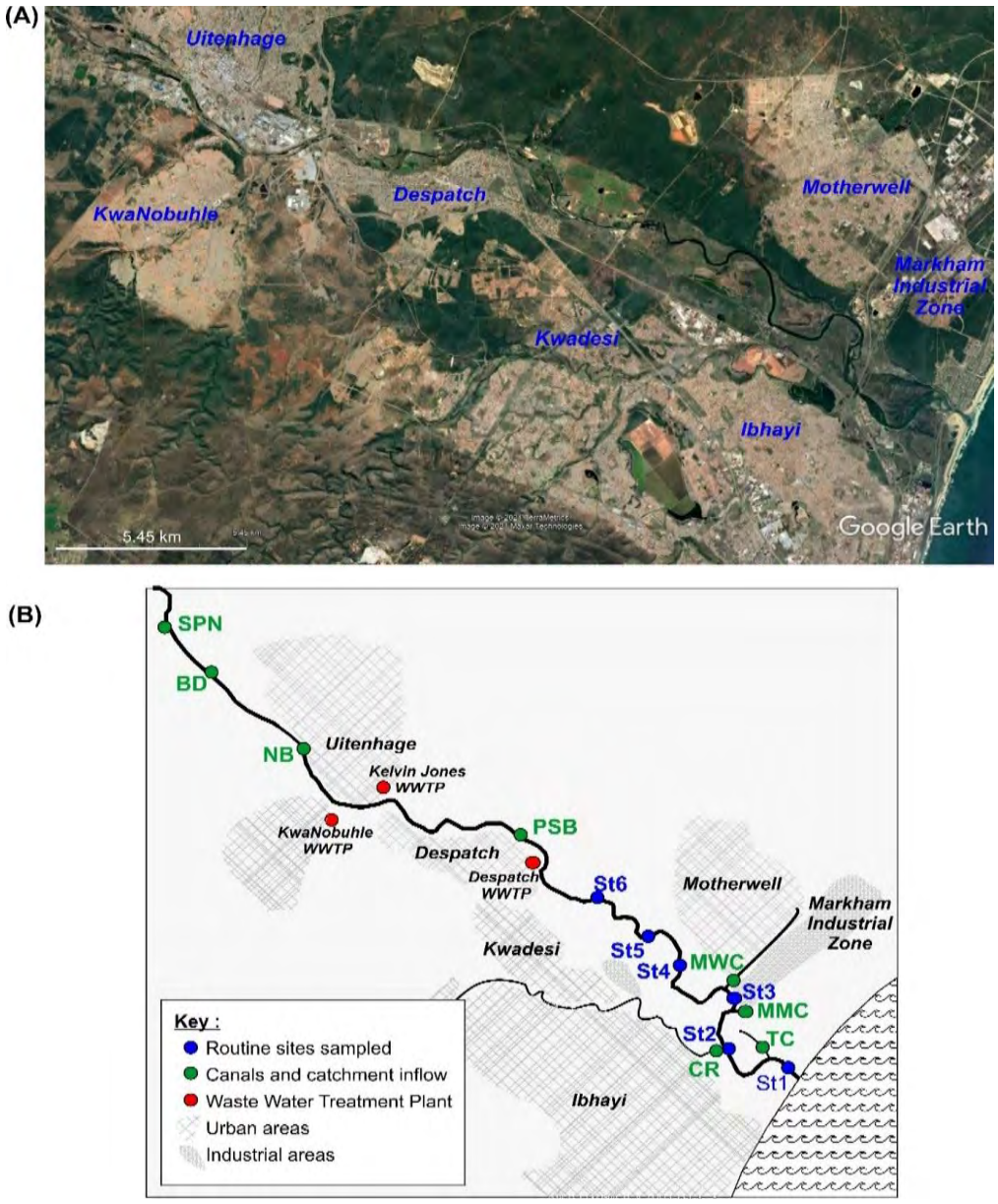

Figure 5.1. Aerial view **(A)** and a geographical map **(B)** of sampling sites along the length of the Swartkops Estuary and potential sources of pollution and discharging points of pollutants in the estuary. Satellite Imagery: Google Earth Maps. (TC – Tippers creek, CR – Chatty river, MMC – Markman canal, MWC – Motherwell canal, PSB – Perseverance bridge, NB – Nivens bridge, BD – Bulmers drift, SPN – Springfontein).

The Motherwell canal also runs through informal settlements (Motherwell township) (Figure 5.1) where illegal dumping takes place and litter, and other pollutants, are directly dumped into the

canal or enter the canal through drains. Due to the high rate of pollution from anthropogenic inputs around the Motherwell canal, a wetland was constructed at the lower side of the canal to reduce the concentration of pollutants entering the estuary (Nel *et al*., 2015). While effective to a large degree, the inflow into the wetland exceeds the capacity of the wetland to remove pollutants completely which end up in the estuary. The stormwater from fourteen drains that are connected to the canal discharge polluted stormwater which passes through litter traps before reaching the wetland (Adams *et al*., 2019; Nel *et al*., 2020). The litter traps sometimes get overloaded, and pollutants pass through the canal into the estuary. The Motherwell canal is frequently affected by the overflow of raw sewage from the residential Motherwell area. The samples taken for this study from the Motherwell canal were taken from the water outflow after the artificial wetland and just prior to entering the estuary. The Markman canal is in proximity to industrial sites and is connected to various stormwater drains that discharge industrial effluents into the canal. It flows through a semi-urban area where there are poorly maintained sewage pumps that frequently break down and discharge raw sewage into the canal (Adams *et al*., 2019).

Swartkops river passes through the western side of Uitenhage and east side of Despatch which are residential areas (Figure 5.1) and have industries that are potential sources of pollution in the estuary. There is illegal dumping around the Uitenhage area which possibly impacts the Swartkops river and estuary (Adams *et al*., 2019). Swartkops river discharges pollutants into Swartkops Estuary from its tributaries such as the Elands river situated on the south side of the river and Kwa-Zunga situated on the north side of the river (Maclear. 1996). Due to the high salinity in Swartkops river, there are limited agricultural activities taking place around Uitenhage, KwaNobuhle and Despatch areas (Baird *et al*., 1986). The Swartkops river had four sampling sites namely, Perseverance bridge (PSB), Nivens bridge (NB), Bulmers drift (BD), and Springfontein (SPN) (Figure 5.1). The Perseverance bridge is located close to the Despatch wastewater treatment works, a residential area, and a gravel quarry (Adams *et al*., 2019). The Kelvin Jones and KwaNobuhle wastewater treatment plants are situated close to the river, upstream of Perseverance bridge (Figure 5.1). KwaNobuhle wastewater treatment plant discharges effluents

via the Brak river that carries stormwater from the surrounding residential areas into the Swartkops river (Adams *et al*.,2019). The Nivens bridge is situated upstream of Perseverance bridge and stormwater from the townships enters the Swartkops river via the Kat canal and passes through the Nivens bridge towards the estuary. The Bulmers drift and Springfontein are situated upstream in the Swartkops river, where freshwater flows through towards the lower side of the river and into the estuary. The waters around the Bulmers drift and Springfontein have previously been reported to be pristine (Bate *et al*., 2004).

5.2 Physico-chemical characteristics of the water channels

The activities taking place along the waterways flowing into the Swartkops Estuary impacted the physical condition of the water (Figure 5.2). When comparing the physical variables, salinity, dissolved oxygen concentration, and turbidity displayed a noticeable difference (Figure 5.2), while there was little variation in temperature and pH (Appendix 3; Table S13).

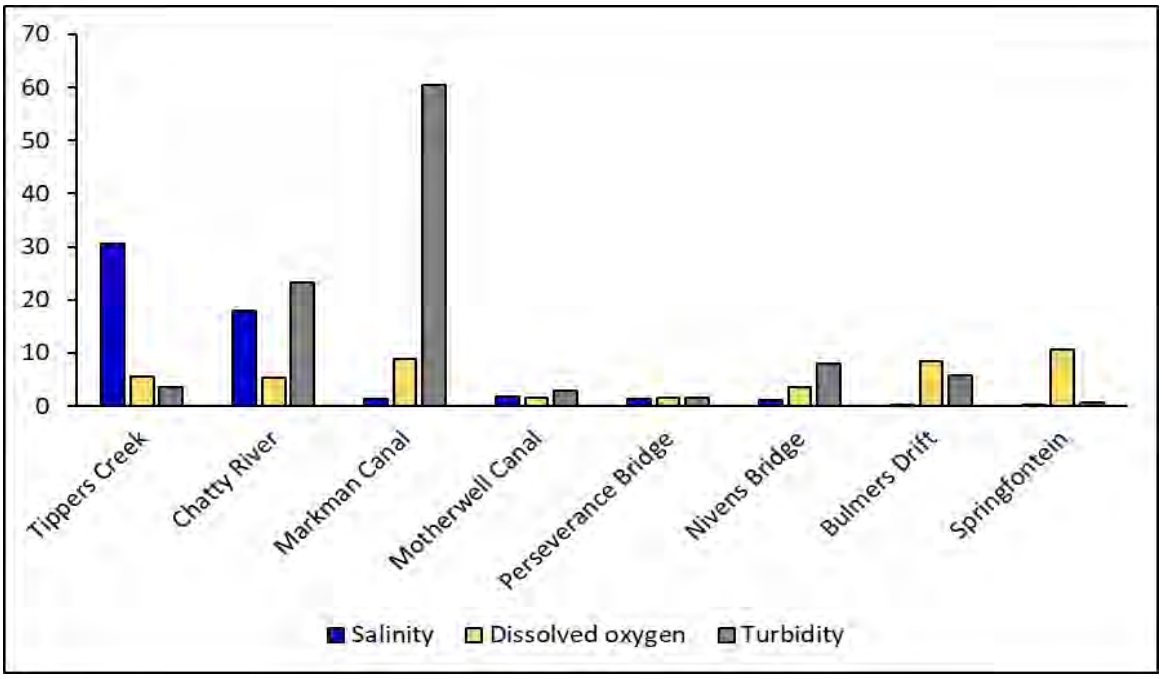

Figure 5.2: Salinity (ppt), dissolved oxygen (mg/L) and turbidity (FNU) measured from canals, rivers and creek during sampling in autumn (2019)

Tippers creek and Chatty river, which discharge water to the lower reaches, had high salinity of > 17 ppt, probably due to incoming tidal waters from the ocean as they are situated close to the mouth of the estuary, while other sampling points were fresh (< 2 ppt) (Figure 5.2). Springfontein and Bulmers drift, situated upstream in Swartkops river, recorded the lowest salinities of 0.11 and 0.29 ppt, respectively. However, the two sampling points together with Markman canal recorded higher dissolved oxygen concentrations compared to other waterways flowing into Swartkops Estuary (Figure 5.2). When comparing the percentage of dissolved oxygen to the freshwater quality standards as outlined by the (Department of Water Affairs and Forestry, 1996), only Bulmers drift, Springfontein, and Markman canal were in good conditions (80 -~120%) (Appendix 3, Table S13). Since water in Tippers creek and Chatty river matched the salinity of estuarine systems, their measure of dissolved oxygen was compared against the water quality standards of estuaries which classed them as well-oxygenated (>5 mg/L) (Lemley *et al*., 2015).

Turbidity was noticeably high in Markman canal and Chatty river (Figure 5.2) which was probably due to the continuous discharge from industries, faulty sewage treatment works, or resuspension of sediments as the water flowed towards the estuary, while other sites recorded turbidity of < 8 FNU.

Nutrient concentrations also varied between the waterways flowing into Swartkops Estuary (Figure 5.3). Canals discharging water into the middle reaches of the estuary (Motherwell and Markman canals) had high total nutrient concentrations with a high concentration of TOxN and ammonium in the Motherwell canal (Figure 5.3). The water in this canal was classed as hypertrophic (> 10,000 µmol/L) while Markman canal and Chatty river were eutrophic in terms of nitrogen levels (in TOxN) in freshwater (Department of Water Affairs and Forestry, 1996) and estuaries (> 1,000 µmol/L), respectively (Lemley *et al*., 2015). Bulmers drift, Springfontein and, Tippers creek had lower nutrients concentrations than in other water channels. Bulmers drift and Springfontein were in good conditions (< 500 µmol/L) in terms of nitrogen levels in freshwater

(Department of Water Affairs and Forestry, 1996) while Tippers creek was mesotrophic (≥100 but <1,000 µmol/L) in terms of nitrogen levels in estuaries (Lemley *et al*., 2015).

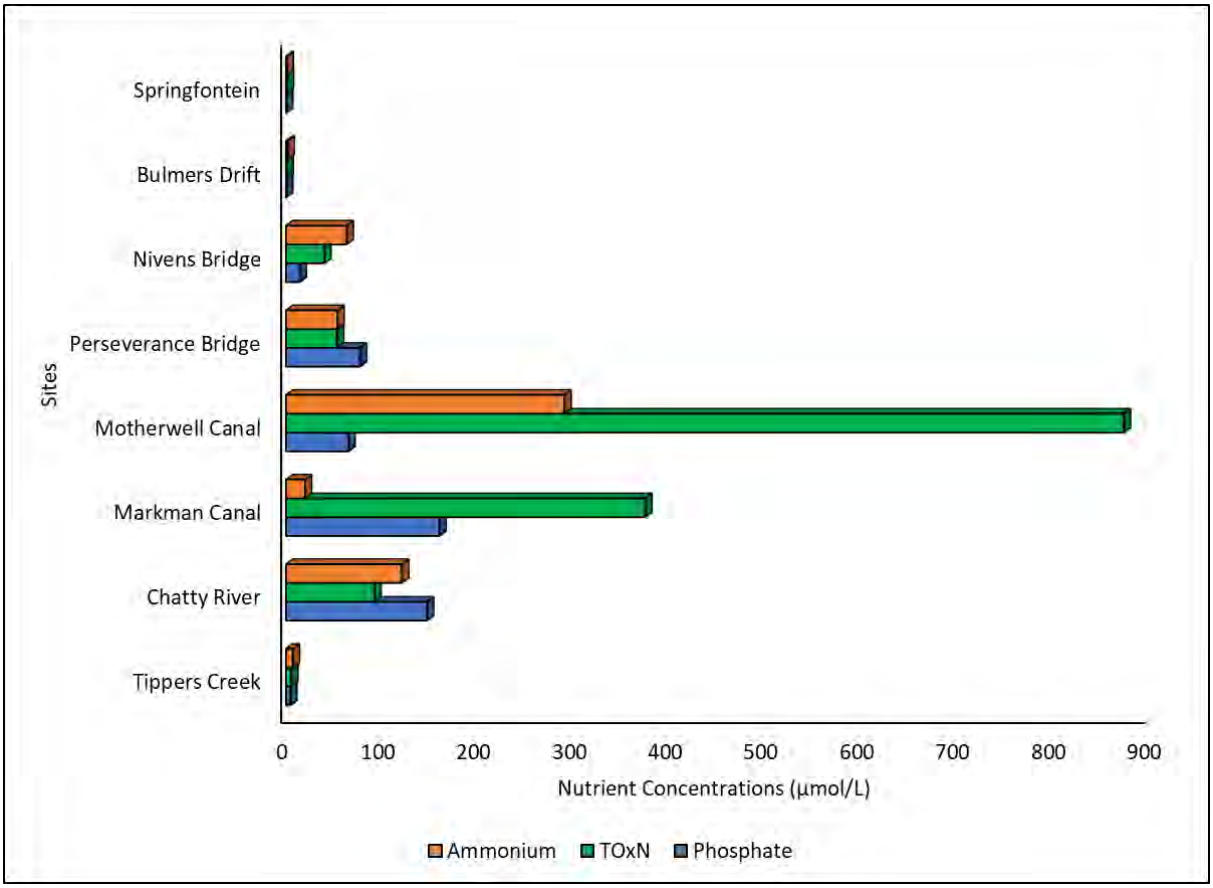

Figure 5.3: Nutrient concentrations of samples collected from canals, rivers and creek flowing into Swartkops Estuary in autumn (2019).

Markman canal and Chatty river had high concentrations of phosphates, which measured 160 µmol/L and 147 µmol/L, respectively, at the time of sampling (Figure 5.3). Most of the water channels were considered as major conduits of phosphorus to Swartkops Estuary, as the amount of phosphorus (in phosphates) exceeded the standard threshold levels of water quality (> 5 µmol/L). An exception was Springfontein which was in good conditions (< 5 µmol/L) in terms of phosphorus levels in freshwater (Department of Water Affairs and Forestry, 1996).

5.3 Bacterial communities - Alpha Diversity

The average number of bacterial OTUs in each sampling site was plotted in rarefaction curves (Figure 5.4). All the rarefaction curves started to approach asymptote indicating that sampling and sequencing depth were sufficient to identify most bacterial OTUs in the river, canals, and creek flowing into Swartkops Estuary. This does not mean that all rare species were represented in the dataset, therefore, it was important to carry out further analysis (Figure 5.5 – Chao1) that accounts for/or considers rare species richness.

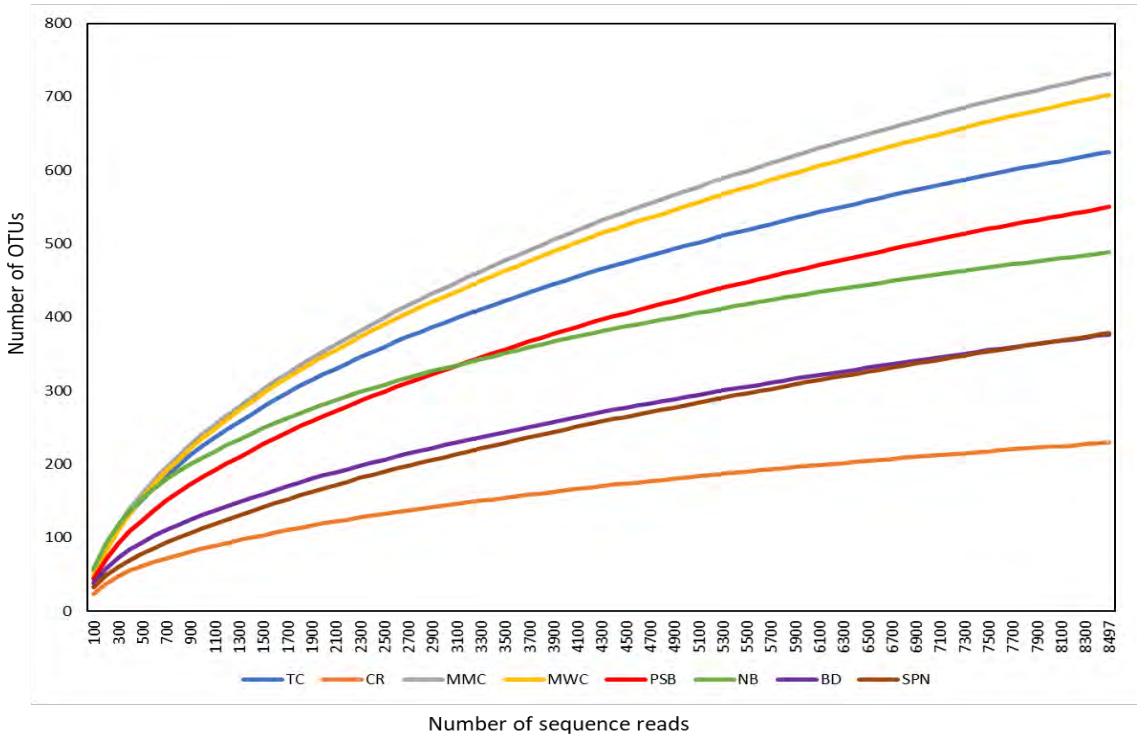

Figure 5.4: Rarefaction curves of bacterial OTUs (generated at a distance of 0.03) from water samples collected from water channels discharging into Swartkops Estuary in autumn (2019). Samples were subsampled at 8497 sequences.

Despite the rarefaction curves not reaching complete asymptote, Chao1 indicated that rare species were well represented in the dataset with only small increased in species richness when considering observed OTUs vs. Chao1 for many of the samples (Figure 5.5).

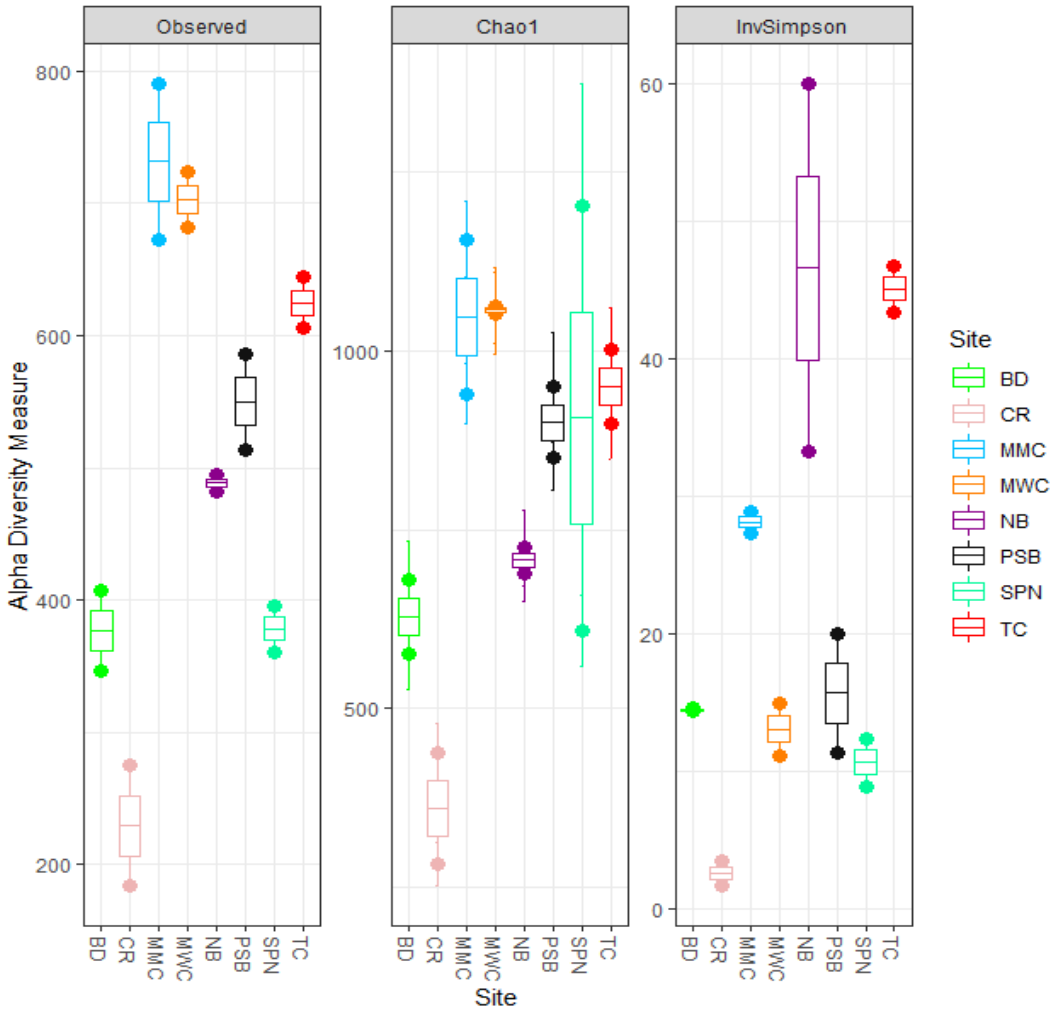

Figure 5.5: Species richness estimator (Chao1) and species richness and diversity (InvSimpson) of the bacterial communities in the water channels flowing into Swartkops Estuary during sampling in the autumn season (2019)

The lowest species richness was observed in the Chatty river with Bulmers drift and Nivens bridge samples exhibiting intermediate richness (Figure 5.5). The remaining samples all showed higher species richness values which were more in line with what was observed for the Swartkops Estuary sites (Chapter 4, Figure 4.6). It should be noted that the Chao1 values for the replicate samples from Springfontein varied greatly. This may be due to the shallow nature of the river at this point (<0.5 m) and some of the sediment may have been kicked up and co-sampled with the water at the time of sampling. In terms of species diversity (InvSimpson, Figure 5.5), the Chatty river samples had the lowest diversity whilst Nivens bridge and Tippers creek had the highest

InvSimpson values corresponding to higher diversity. The Bulmers drift, Motherwell canal, Perseverance bridge, and Springfontein exhibited similar levels of bacterial diversity (Figure 5.5).

5.4 Taxonomic classification

Bacterial communities and abundances varied greatly between water channels and reflect the anthropogenic variables each are subjected to (Figure 5.6, Appendix 3; Table S15). The results in Figure 5.3 shows that 7 out of 8 water channels were potential conduits of phosphorus into Swartkops Estuary. Not surprisingly, ANOSIM analysis with phosphorus levels as the variable resulted in an ANOSIM global test analysis with significant differences between eutrophic, mesotrophic and oligotrophic sampling sites (p-value of 0.001 and an R-value of 0.712) (Appendix 3; Table S14).

The inflow channels bacterial communities were very different to that of the estuary. Bacterial families that occurred in high relative abundances in the water channels and were either non-dominant or low in relative abundances in the estuary which included members of *Phormidiaceae, Arcobacteraceae, Aeromonadaceae, Alteromonadaceae, Chitinophagaceae, and Spirosomaceae. Phormidiaceae* were predominant in Chatty river, contributing 98.8% of its sequences in this dataset, while *Arcobacteraceae, Aeromonadaceae,* and *Alteromonadaceae* were in high relative abundance in the Motherwell canal. Members of *Spirosomaceae* were in high relative abundance in Bulmers drift contributing 72.8% of its sequences in this dataset (Figure 5.6) and were absent/low in relative abundance in other water channels and estuary. Some bacterial taxa such as the Alphaproteobacteria were prevalent in the Swartkops Estuary while they were in low relative abundance in point sources with the exception of Tippers creek (Figure 5.6).

The inflow from the water channels had an impact on bacterial community composition in Swartkops Estuary. This was reflected by the presence of bacterial communities which are not

natural inhabitants of estuarine systems, such as freshwater inhabitants and pathogenic bacteria, in the water channels and sampling sites within the estuary adjacent to inflow entry points. Members of *Burkholderiaceae* which were in high relative abundance in sampling sites situated in Swartkops river, (Springfontein, Bulmers drift, Nivens bridge, and Perseverance bridge, in descending order of closer proximity to the estuary), were identified on the water surface of the upper reaches of the estuary (Figure 5.6). *Burkholderiaceae* decreased in relative abundance downstream and down to the bottom of the water column. The canals and Chatty river also had a high relative abundance of *Burkholderiaceae* which were also found in sampling points in the estuary adjacent to the entry points (sites 2 and 3).

Other bacterial communities that may have entered the estuary from the catchment include members of *Rhodocyclaceae* (Gammaproteobacteria) which were found in high relative abundance in Nivens and Perseverance bridge and water surface of the upper reaches of the estuary and decreased downstream and down the water column (Figure 5.6). *Rhodocyclaceae* were also found in high relative abundance in Markman canal, however, they were in low relative abundance in site 2 which was the closest sampling site in the estuary.

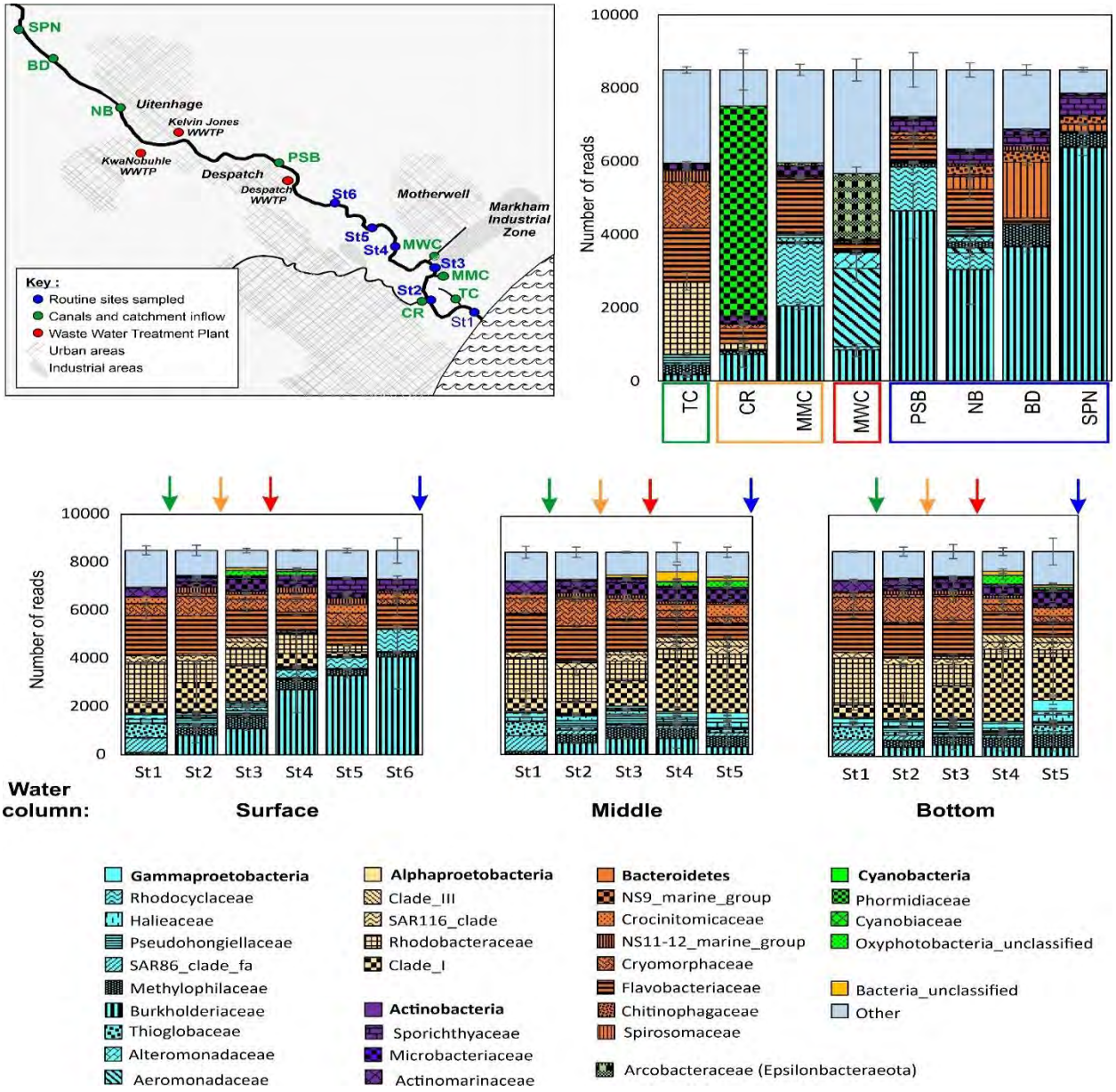

Figure 5.6: Phylogenetic characterization at the taxon level of family showing the inflow of bacterial communities from the canals, rivers, and creek into Swartkops Estuary during sampling in autumn 2019. Error bars represent standard deviation of each family taxon in each sampling site (n=2). The arrows indicate the point of inflow from the channels relative to the estuary sampling sites (the colors correspond to the grouping of the inflow channels). (TC: Tippers creek; CR: Chatty river; MMC: Markman canal: MWC: Motherwell canal; PSB: Perseverance bridge; NB: Nivens bridge; BD: Bulmers drift; SPN: Springfontein)

Of the water inflow points, Tippers creek and to a lesser extent Markman canal exhibited bacterial community profiles most similar to that of the estuary. This is due to the fact that both sites are located closer to the mouth of the estuary and are heavily influenced by tidal action as evidenced by the salinity readings (polyhaline) at the time of sampling (Figure 5.2), which means that much of the bacterial community present at this site may have been from the ocean. Bacterial communities that were in high relative abundance in Tippers creek were also in high relative abundance at the lower reaches of the estuary. These include members of *Cryomorphaceae, Flavobacteriaceae,* and *Rhodobacteriaceae*. The Alphaproteobacteria were prevalent in the Swartkops Estuary while they were in low relative abundance in point sources with the exception of Tippers Creek (Figure 5.6).

5.4.1 Bacterial OTUs

The OTUs presented in Figure 5.7 are a reflection of the shared OTUs between the point sources and sampling sites in the estuary. The dominant OTUs were sorted similarly to what was described in Chapter 3 and were subjected to BLASTn analysis against the GenBank NCBI nr/nt and Refseq_rna databases (Table 5.1). A more detailed heatmap of the dominant OTUs can be found in appendix 3. Due to the salt wedge effect, where freshwater flows over marine water, the water surface in the estuary provided a better reflection of bacterial OTUs that entered the estuary than the middle and bottom of the water column. Consequently, the results of this section were focused on the water surface since the water from the channels was freshwater.

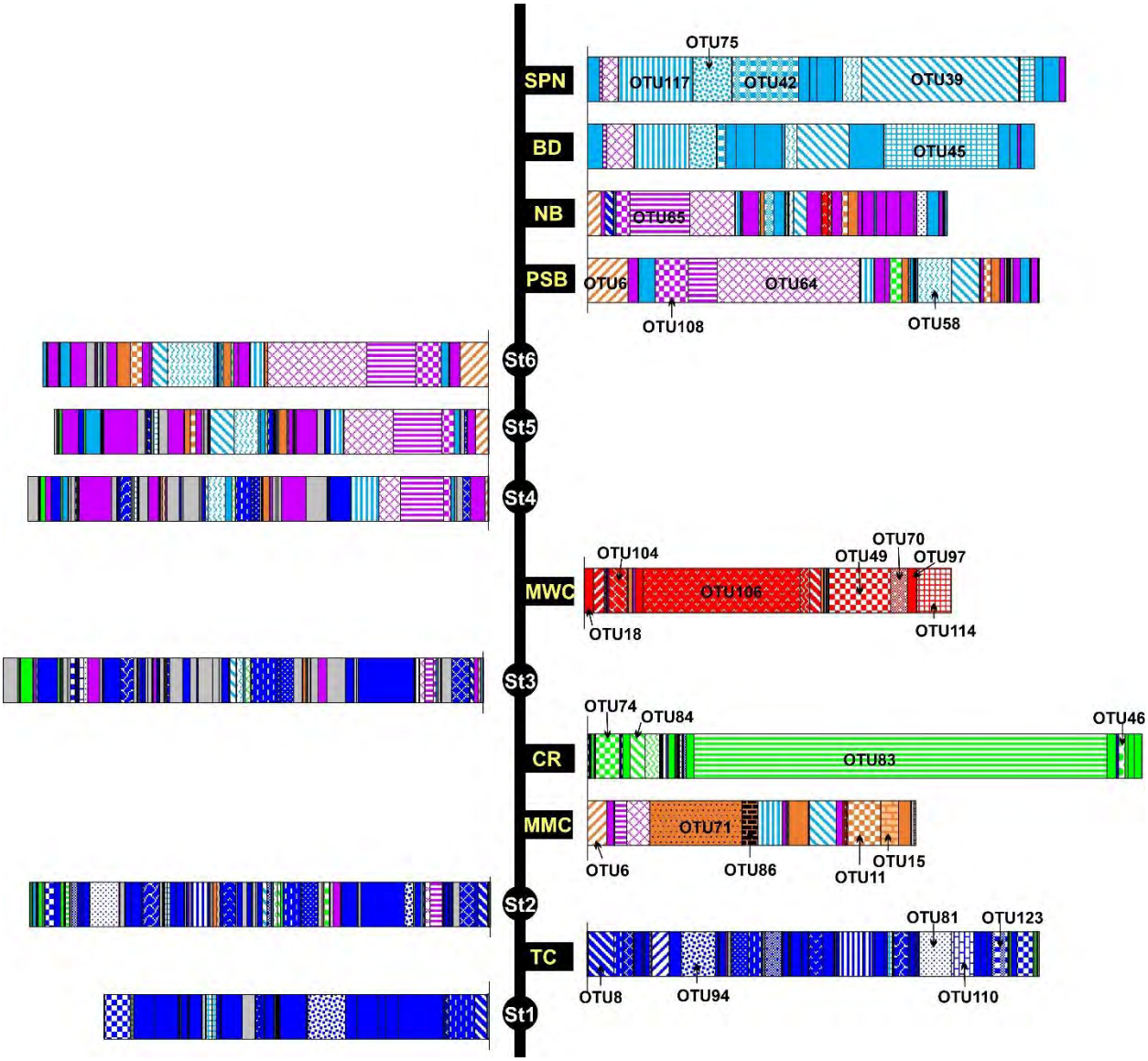

Figure 5.7: Dominant OTUs that entered the surface waters of Swartkops Estuary from the canals, rivers and creek. (Light blue color = Dominant OTUS identified in Springfontein (SPN) and Bulmers drift (BD), Pink = Dominant OTUs from Nivens bridge (NB) and Perseverance bridge (PSB), Red = Dominant OTUs from Motherwell canal (MWC), Green = Dominant OTUs from Chatty river (CR), Orange = Dominant OTUs from Markman canal (MMC), Dark blue= Dominant OTUs from Tippers creek (TC).)

Table 5.1: Blast match results of bacterial OTUs at genus/species level against the GenBank database and Silva_v132 reference database

OTU	Classification against the silva_v132 database	Blast analysis against the GenBank nr/nt database			Blast analysis against the GenBank Refseq RNA database		
		Accession number	Identity match %	Closest match in NCBI	Accession number	Identity match %	Closest match in NCBI
OTU6	C39	LC132836.1	98.06%	*Rhodocyclaceae*	NR_028678.1	93.24%	*Azovibrio restrictus*
OTU8	*Rhodobacteraceae*_unclassified	KP262720.1	98.54%	Uncultured bacterium	NR_164623.1	97.08%	*Aliishimia ponticola*
OTU11	*Flavobacterium*	JQ177676.1	99.02%	Uncultured *Flavobacterium*	NR_118478.1	98.03%	*Flavobacterium siccinicans*
OTU15	*Flavobacterium*	KM141914.1	98%	Uncultured bacterium	NR_118476.1	97.54%	*Flavobacterium hydatis*
OTU18	*Shewanella*	CU467450.1	98.79%	Uncultured bacterium	NR_113582.1	98.54%	*Shewanella putrefaciens*
OTU39	*Polynucleobacter*	MT066688.1	99.27%	Uncultured bacterium	NR_151918.1	98.78%	*Polynucleobacter duraquae*
OTU42	Clade_III_ge	KP708781.1	99.03%	Uncultured bacterium	NR_116131.1	98.05%	*Acidovorax delafieldii*
OTU45	*Pseudarcicella*	FN668111.2	99.01%	Uncultured *Flectobacillus*	NR_165029.1	98.76%	*Aquirufa antheringensis*
OTU46	PeM15_ge	LR641432.1	99.28%	Uncultured bacterium	NR_159886.1	95.41%	*Longivirga aurantiaca*
OTU49	*Arcobacter*	AB205716.1	98.79%	Uncultured bacterium	NR_042218.1	99%	*Arcobacter cibarius*
OTU58	*Burkholderiaceae_unclassified*	MT239563.1	98.30%	*Curvibacter* sp.	NR_125544.1	98.05%	*Limnohabitans australis*
OTU64	*Burkholderiaceae*_unclassified	KM163175.1	98.78%	Uncultured bacterium	NR_125491.1	98.54%	*Limnohabitans curvus*
OTU65	*Burkholderiaceae*_unclassified	HF968558.1	99.51%	Uncultured *Limnohabitans*	NR_125541.1	99.27%	*Limnohabitans planktonicus*
OTU70	*Arcobacter*	JX912354.1	99.01%	Uncultured *Arcobacter*	NR_117570.1	98.52%	*Arcobacter cloacae*
OTU71	C39	LC132788.1	99.03%	*Rhodocyclaceae bacterium*	NR_028678.1	96.61%	*Azovibrio restrictus*
OTU74	*Burkholderiaceae*_unclassified	KX163849.1	99.03%	Uncultured bacterium	NR_158145.1	98.78%	*Hydrogenophaga soli*
OTU75	*Burkholderiaceae_unclassified*	CP054840.1	99.03%	*Acidovorax* sp.	NR_113696.1	98.78%	*Curvibacter delicatus*
OTU81	Uncultured *Cryomorphaceae*	MF498311.1	99.02%	Uncultured bacterium	NR_136475.1	92.44%	*Phaeocystidibacter marisrubri*
OTU83	*Arthrospira_PCC-7345*	MT426015.1	98.31%	*Arthrospira platensis*	NR_125711.1	97.58%	*Arthrospira platensis*
OTU84	*Idiomarina*	MN746254	98.31%	*Idiomarina aestuarii*	NR_116804.1	97.34%	*Idiomarina aestuarii*

OTU86	*Thiobacillus*	GQ860185.1	99.27%	Uncultured bacterium	NR_115758.1	98.31%	*Thiobacillus sajanensis*
OTU94	*Amylibacter*	JQ194950.1	98.78%	Uncultured bacterium	NR_146351.1	97.81%	*Amylibacter ulvae*
OTU97	*Arcobacter*	JQ754668.1	99.01%	Uncultured *Arcobacter* sp.	NR_117105.1	98.28%	*Arcobacter ellisii*
OTU104	*Acinetobacter*	KR072675.1	99.51%	*Acinetobacter sp. Lam-1*	NR_165666.1	99.27%	*Acinetobacter chinensis*
OTU106	*Aeromonas*	MT254904.1	99.30%	*Aeromonas Enteropelogenes*	NR_116586.1	99.03%	*Aeromonas Fluvialis*
OTU108	C39	EU234274.2	98.55%	Uncultured bacterium	NR_028678.1	94.44%	*Azovibrio restrictus*
OTU110	Uncultured *Cryomorphaceae*	KF917687.1	98.29%	Uncultured bacterium	NR_136475.1	91.48%	*Phaeocystidibacter marisrubri*
OTU114	*Arcobacter*	CP030944.1	98.54%	*Arcobacter aquimarinus*	NR_136421.1	98.54%	*Arcobacter aquimarinus*
OTU117	*Polynucleobacter*	HQ111147	98.54%	Uncultured *Polynucleobacter*	NR_151920.1	98.30%	*Polynucleobacter sinensis*
OTU123	NS3a_marine_group	KU173779.1	98.79%	Uncultured *Chlorobi* group bacterium	NR_025749.1	93.72%	*Maribacter orientalis*

The water inflow from the Swartkops river discharged a higher proportion of bacterial OTUs into the estuary compared to water discharged by other point sources (Figure 5.7 Figure 5.8). The bacterial OTUs from the sampling points upstream in the Swartkops river (i.e. Springfontein (SPN) and Bulmers drift (BD)) decreased in relative abundance as the water flowed past the Nivens bridge and Perseverance bridge and into the estuary. This was shown by OTU117 and OTU39 with sequence similarity to *Polynucleobacter* sp. (Table 5.1) which were in high relative abundance in Springfontein and decreased to Bulmers drift and occurred in different degrees of abundance as they flowed past the Perseverance bridge into the estuary. Perseverance bridge had a high abundance of OTU58 (98.3% *Curvibacter* sp.) which was low in relative abundance at other sampling sites in Swartkops river and other water channels. It was also identified in high abundance at site 6 and decreased down the estuary. These OTUs (OTUs 117, 39, 58) were noticeably abundant in the estuary up to site 4 and were very low in relative abundance or absent at sites at the mouth of the estuary. Even though Swartkops river shared most of the OTUs with the estuary, there were distinct differences such as OTU42 (98% sequence similarity to *Acidovorax delafieldii*) and OTU45 (98.8% sequence similarity to *Aquirufa antheringensis*) which were numerically abundant in Springfontein and Bulmers drift respectively and were absent/very low in relative abundance in the estuary. OTU75 (99% sequence similarity to *Acidovorax* sp.) was numerically abundant in Springfontein and decreased in relative abundance at Bulmers drift and was absent/low in relative abundance in the estuary.

OTU64 (98.5% sequence similarity to *Limnohabitans curvus*) occurred in increased abundances in sampling sites in the river as the water flowed towards the estuary and as it entered the estuary, it decreased in relative abundance towards the lower reaches (Figure 5.7, Table 5.1). Nivens bridge and Perseverance bridge had a high relative abundance of OTU65 (99% sequence similarity to *Limnohabitans planktonicus*) and OTU108 and OTU6 were numerically abundant in Perseverance bridge. These OTUs (OTUs 65, 108, 6) were also present in the estuary. OTU65 was prevalent at sites 6 – 4 and distributed in low relative abundance to the lower reaches of the estuary while OTU108 and OTU6 occurred in decreased abundances from the upper to the lower reaches. The Markman canal, which is heavily influenced by industries, and the Swartkops river

which is also situated closer to industries and both run close to residential areas, had abundance of similar bacterial OTUs such as OTU65, OTU64, OTU117, OTU39, and OTU6. Markman canal also had a high relative abundance of OTU71 (sequence similarity to C39), OTU11 (98% sequence similarity to *Flavobacterium succinicans*), OTU86 (98% sequence similarity to *Thiobacillus sajanensis*), and OTU15 (97.5% sequence similarity to *Flavobacterium hydatis*) which were absent/very low in abundance in the estuary (Figure 5.7, Table 5.1).

Not surprisingly, since the Motherwell canal is well known for illegal dumping and inflow of raw sewage from poorly maintained treatment plants, most of the OTUs identified in this canal had sequence similarity to feacal and/or pathogenic bacteria including OTU106 (99% sequence similarity to *Aeromonas enteropelogenes*), OTU104 with 99.27% sequence similarity to *Acinetobacter chinensis,* as well as OTUs showing sequence similarity to *Arcobacter* sp. (OTUs 49, 114, 70 and 97) (Figure 5.7, Table 5.1). OTU18 (sequence similarity to *Shewanella* sp.) was also identified in the canal. These OTUs were absent or in low relative abundances in the estuary which may be due to moderately low pressure from the canal or inability to survive in estuarine conditions. Alternatively, and most likely, the high bacterial loads in the estuary waters resulted in the low relative abundances of inflowing bacterial cells from the Motherwell canal even though their absolute cell numbers remained constant.

The Chatty river (CR), which discharges into the lower reaches of the estuary from densely populated residential areas, had a numerical abundance of OTU83 which was also observed in very low relative abundance at site 2, close to the entry point of the water inflow (Figure 5.7, Table 5.1). OTU74 (98.8*%* sequence similarity to *Hydrogenophaga soli*), OTU84 (97% sequence similarity to *Idiomarina*), and OTU46 were identified in Chatty river and in very low relative abundance at site 2. OTU74 was also present in low relative abundance in Perseverance bridge. Though Tippers creek was almost similar to site 1 at the taxon level of family, there are noted distinct differences such as OTU110 and OTU123 which were in high relative abundance in the creek but absent in the estuary. OTU8 (97% sequence similarity to *Aliishimia ponticola*), OTU94

(97.8% sequence similarity to *Amylibacter ulvae*), and OTU81 which did not share any significant sequence similarity to any genera in the NCBI reference database, were in high relative abundance in Tippers creek and present at the lower reaches of the estuary (Figure 5.7, Table 5.1).

The inflow from Tippers creek (TC) is discharged at proximity to the mouth of the estuary (site 1) and the Chatty river (CR) and Markman canal (MMC) flow into the estuary closer to site 2 (Figure 5.1). Despite the fact that at a family level, Tippers creek looked very similar to site 1, only 6,52% of the OTUS from site 1 were also found in Tippers creek (Figure 5.8A). Of the OTUs assigned to site 2, 2.01% and 2.74% were also present in the adjacent catchment inflow sites to site 2, namely Chatty river and Markman canal, respectively (Figure 5.8B). None of the OTUs were common between all three sites. The Motherwell canal, which discharges upstream of site 3, had the most number of unique OTUs (98%) and ~2% of the OTUs in the Motherwell canal dataset were found in the estuary dataset (Figure 5.8C).

The closest sampling point in Swartkops river flowing into site 6 at the upper reaches of the estuary was Perseverance bridge (Figure 5.1), which had 9.6% of its bacterial OTUs in the estuary (Figure 5.8D). The shared OTUs between site 6 and Swartkops river inflow (Perseverance bridge, Nivens bridge, Bulmers drift, and Springfontein) decreased numerically with increasing distance from the estuary due to changing physico-chemical parameters and dilution as the water flows downstream. This was also observed in Figure 5.8E where the number of shared OTUs between the river inflow sites and the estuary decreased numerically as the water flows downstream to site 4. Since the sampling sites 4 and 5 were not adjacent to any sampled channel inflow (Figure 5.1), the number of OTUs shared between the Swartkops river inflow and site 4 (middle reaches) (Figure 5.8E) indicates the extent of pollution from the river inflow. Only 5.6% of the OTUs from Swartkops river were also found within the estuary (sites 4-6).

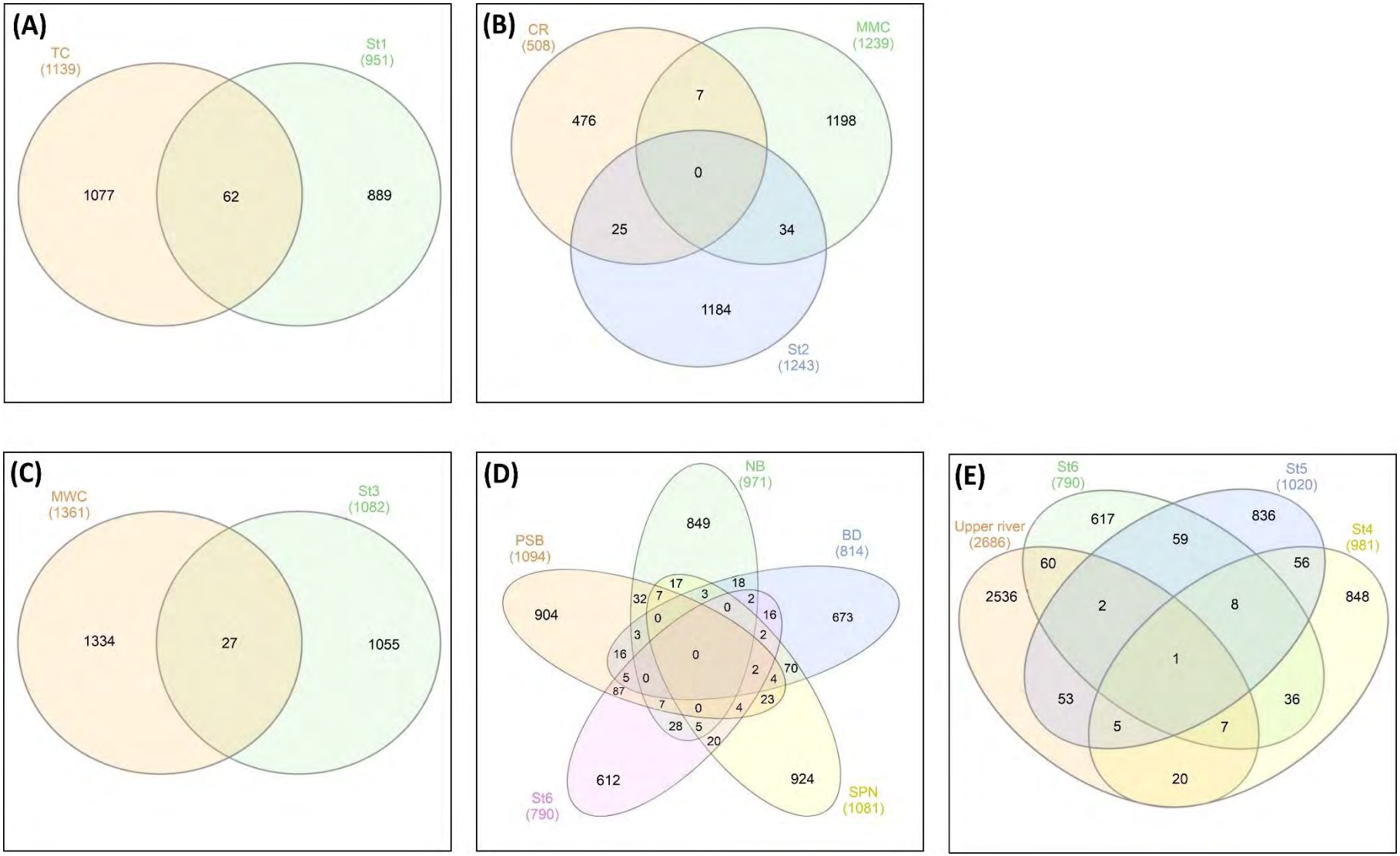

Figure 5.8: Comparative Venn Diagrams analysis of bacterial OTUs from the water surface of Swartkops Estuary and water inflow from canals, rivers, and creek. OTUs were generated using unsubsampled datasets. Figures were generated using InteractiVenn Software (Heberle *et al.,* 2015)

Overall, only a small percentage of the bacterial OTUs present in the inflow water bodies datasets were also present in the datasets representing the estuary itself. This does not exclude the possibility of an increased overlap in OTU representation between these two datasets if additional sequencing to a greater sequence depth was conducted (as evidenced in Chapter 4; Figures 4.5 and 5.4, not all of the rare OTUs were present in the respective datasets). This was also reflected at the family level in Figure 5.6 where a sizeable number of bacterial families from the inflow were non-dominant in the estuary.

5.4.2 Potential Pathogens

As discussed in the introduction, contamination of the Swartkops Estuary from animal carcasses, poorly maintained/managed wastewater treatment plants and raw sewage input from runoff from informal settlements does occur and as a result, the potential for occurrence of human pathogenic bacteria in these water bodies is of great concern. The 16S rRNA sequences of potential waterborne pathogens were extracted from the nucleotide NCBI database and used to create a custom reference database (Appendix 3; Table S16). Bacterial sequences from the dataset were then subjected to BLAST analysis against the newly created database to identify potential pathogens in both the water channels and Swartkops Estuary datasets. Only those that showed >99% sequence similarity to bacterial pathogens were represented in Figure 5.9. The presence and abundance of these bacterial pathogens indicate how urbanization has affected the health status of the estuary and impacted the bacterial structure in both the estuary and water channels. Sampling from the rivers, canals and creek was done once during the autumn season and the presence of bacterial pathogens in the estuary at the time of sampling during other seasons is indicative of consistent discharge of pathogens by the water channels.

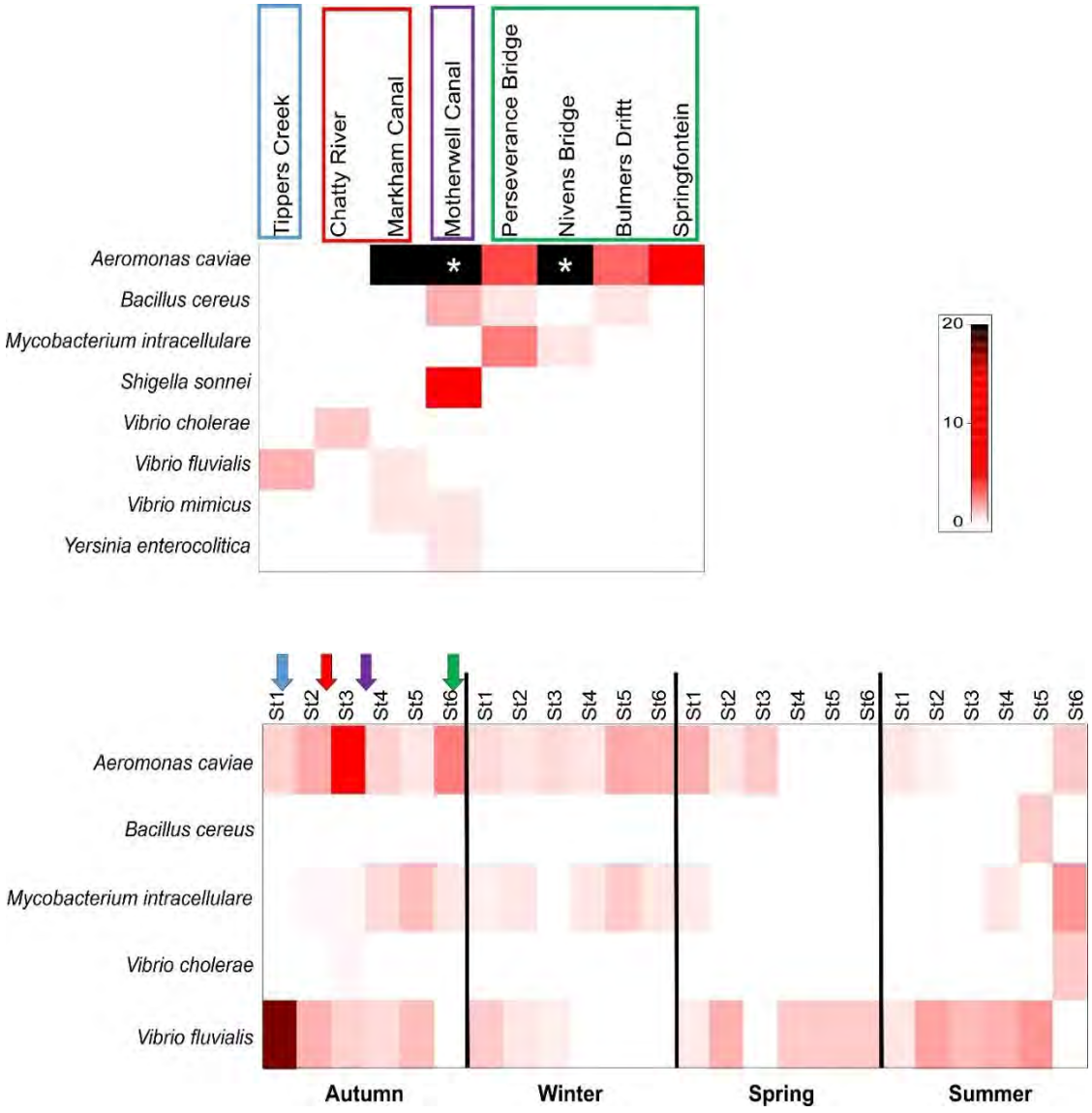

Figure 5.9: Putative bacterial pathogens from the canals, rivers and creek flowing into the Swartkops Estuary. Arrows indicate the inflow points relative to the sample sites within the estuary with the colors corresponding to those of the outlines bordering the labels of water channels.

When comparing bacterial pollution between the sampled water channels, the Motherwell canal was the most polluted with bacterial pathogens (Figure 5.9) as was also indicated in Figure 5.7. Only *Vibrio cholerae, Vibrio fluvialis,* and *Mycobacterium intracellulare* were absent in the Motherwell canal. Although *Shigella sonnei, Vibrio mimicus,* and *Yersinia enterolitica* were

present in the Motherwell canal, they were absent in the estuary dataset. *Vibrio mimicus* was also present in low abundance in Markman canal.

The canals and Swartkops river (PSB, NB, BD, SPN) had a high abundance of *Aeromonas caviae* which was most abundant in the Motherwell canal (Figure 5.9). Bacterial pollution in the estuary from the water channels was evident with the high relative abundances of *Aeromonas caviae* at sites closer to the entry points on the water inflow such as sites 3 and 6 although it distributed along the length of the estuary. *Aeromonas caviae* was also prevalent in the estuary during sampling in winter and present in abundance at different sites of the estuary during sampling in spring and summer. The water channels discharging water into the lower reaches of the estuary had a high relative abundance of *Vibrio* species. The Chatty river had a high prevalence of *Vibrio cholerae* while Tippers creek had high numbers of *Vibrio fluvialis*, which were also present in the estuary. *Vibrio cholerae* was identified at site 6 during sampling in summer while *Vibrio fluvialis* was most numerous at the lower reaches in autumn, where Tippers creek discharges, and was present in most sampling sites along the length of the estuary during sampling in spring and summer but absent/ low in numbers at the upper estuary during sampling in winter. The Perseverance bridge had a large number of *Mycobacterium intracellulare* which was absent in other point sources and occurred in low abundance in Nivens bridge. Although *Mycobacterium intracellulare* was present in most parts of the estuary during sampling in autumn and winter, it was absent in most sampling sites of the estuary during sampling in spring and summer.

5.5 Discussion

Studies conducted in estuaries highlight stormwater inflow as a conduit of pollutants (Sercu *et al.*, 2009; Sauer *et al.*, 2011; Beck & Birch, 2012) which changes the physico-chemical and biological structure of estuarine systems (Crump *et al*., 2004; Fortunato *et al*., 2012). Swartkops Estuary is no exception. The nutrients that commonly contaminate estuaries, namely nitrogen and phosphorus (Birch *et al*., 2010), entered Swartkops Estuary in the form of TOxN and

phosphates via stormwater from canals, creek, and rivers, with the exception of Springfontein which was within the recommended nutrient levels (Figure 5.3). The Motherwell canal was identified as the major source of nitrogen in the Swartkops Estuary in a study by Adams *et al.* (2019), and it was still a major source of nitrogen (TOxN and ammonium) in this study. Nutrient pollution in Swartkops Estuary has reportedly been from overflow of untreated sewage waste due to malfunction or poor maintenance of treatment plants, industrial effluents, and urban runoff which enter the estuary through Swartkops river, canals, and Chatty river (Adams *et al*., 2019). This contributed to variation in bacterial community composition and structure in the canals, rivers, and creek, and estuary. Poorly maintained wastewater treatment works contain high bacterial richness (Liu *et al.*, 2015b) which were observed in the canals (Motherwell and Markman) (Figure 5.5) that are frequently contaminated by raw sewage.

Previous research on the assessment of estuarine microbial water quality focused more on fecal contamination indicators than on microbial communities associated with industrial activities or urban development (Parker *et al*., 2010; Sercu *et al.*, 2011; Sidhu *et al.*, 2012) which played a part in bacterial contamination in Swartkops Estuary. The impacts of the water inflow on bacterial composition in the estuary were evidenced by the presence of similar bacterial communities in point sources and estuary and their distribution along the length of the estuary. With regards to the bacterial communities from the catchment inflow, the proportion of bacterial OTUs that were shared between the catchment inflow (rivers, creek, and canals) and the estuary was low (Figure 5.8). This was likely due to several factors including the inability of freshwater bacteria to survive in estuarine conditions, the flushing of the estuary by tidal events, and a moderate inflow pressure as the accumulative amount of rainfall 5 days prior to sampling was approximately 37.2 mm (as supplied by South African Weather Services). Additionally, estuarine ecosystems are typically very productive with high bacterial biomass (Alongi, 1988; Aguirre *et al*., 2017; Yi *et al*., 2020) and as a result, any input of further bacterial biomass from alternative sources (e.g. canals, river or creeks) would only represent a small proportion of the overall bacterial community.

Burkholderiaceae identified in the dataset were in high relative abundance in the Swartkops river particularly at Springfontein sampling site which was oligotrophic in terms of nitrogen and phosphorus concentration (Figure 5.6). *Rhodocyclaceae* (Gammaproteobacteria) were in high relative abundance in two of the sampling sites in Swartkops river, namely Nivens and Perseverance bridge, as well as in Markman canal where nutrient levels were high. Since *Burkholderiaceae* are well known saprotrophs (Coenye, 2014), their relatively high abundance in the oligotrophic environment may have been from debris or organic matter from the terrestrial environment. Members of this bacterial family (*Burkholderiaceae*) entered the estuary from the upper reaches as indicated by their abundance on the water surface of the upper estuary and a decrease downstream (Figure 5.6). The closest site to receive the inflow from Markman canal (site 2) had a very low relative abundance of *Rhodocyclaceae* which may have been from this canal and the diluted flow from the upper reaches.

There was a distinct difference between Bulmers drift and other potential sources of pollution at the family level. This site had an abundance of *Spirosomaceae* which were absent in other water channels (Figure 5.6). *Spirosomaceae* are freshwater bacteria and can be found in nutrient-rich environments where they degrade complex carbon sources (Li *et al.*, 2017). Although Bulmers drift had very low nitrogen levels, phosphorus levels exceeded the recommended threshold in freshwater. The lower reaches (sites 1 and 2) and Tippers creek had many of the bacterial families in common (i.e. *Rhodobacteraceae, Flavobacteriaceae, Cryomorphaceae*) (Figure 5.6) probably due to tidal influences which was evident with the high salinity in Tippers creek (Figure 5.2). The Chatty river and the canals (Motherwell and Markman) run through densely populated areas and Markman canal is influenced greatly by industries. These three sites were distinctly different in that the Chatty river was dominated by *Phormidiaceae* which was absent in other catchment inflow sites, and Markman canal had a high relative abundance of *Rhodocyclaceae* while *Aeromonadaceae*, *Arcobacteraceae*, and *Thioglobaceae* were high in relative abundance in the Motherwell canal and absent/very low in relative abundance in other catchment inflow sites. Even though the proportion of bacterial communities shared between the water channels and

the estuary was low, many bacterial communities in Swartkops river overlapped with that of the estuary at both family and OTU level compared to other water channels (Figure 5.6, Figure 5.7, Figure 5.8 and Figure 5.9). This could be due to low tidal events at the upper reaches which results in little mixing/low dilution of the river and estuarine water.

The inflow of raw sewage in Swartkops Estuary was shown by fecal contamination indicators that entered the estuary from Swartkops river and the canals. Swartkops river is commonly polluted by overflow of raw sewage from Kwanobuhle, Kelvin Jones, and Despatch wastewater treatment plants while the canals are situated close to sewage pipes that sometimes burst and flow into the canals (Adams *et al*., 2019). These fecal indicators include members of *Arcobacteriaceae, Aeromonadaceae*, and *Rhodocyclaceae* (Figure 5.6) as well as OTUs 6 and 108 with sequence similarity to C39 *(*which is a genus of the *Rhodocyclaceae)* (Figure 5.7, Table 5.1). Members of the *Rhodocyclaceae* commonly inhabit environments with high concentrations of phosphates and oxidized nitrogen (Liu *et al*., 2015a) and it is not surprising that *Rhodocyclaceae* were in high relative abundance in these nutrient-rich environments (Figure 5.6 and Figure 5.7, Table 5.1). *Arcobacter* was identified as an urban signature from sanitary sewer infrastructure in a study by Fischer *et al*. (2015) and *Arcobacter cibarius* was previously identified in wastewater with fecal contamination (Collado *et al*., 2008; Levican *et al.*, 2015; Pérez-Cataluña *et al*., 2018) and from broiler carcass (Houf *et al*., 2005; Yesilmen *et al*., 2017). The presence in high relative abundance of OTU49 with high sequence similarity to *Arcobacter cibarius* in the catchment inflow particularly from the Motherwell canal (Figure 5.7, Table 5.1)*,* confirms the impact of urbanization in Swartkops Estuary.

Members of *Acinetobacter* are also urban signatures as they are associated with pipe infrastructure and were found in abundance in stormwater as non-fecal sewage biomarkers by Fisher *et al.* (2015). They can also be found in soil (Fisher *et al.,* 2015), therefore the terrestrial environment cannot be ruled out as a source of *Acinetobacter* in this study. *Acinetobacter*

are commonly known to remove phosphates from wastewater and, together with *Acidovorax,* were isolated from freshwater systems, hospital environments, vegetables, activated sludge, and wastewater treatment plants (Kämpfer *et al.,* 1996; Ghigliazza *et al.*, 1998; Carr *et al*., 2001; Hu *et al.,*2019). This could account for the high relative abundance of OTU104 with high sequence similarity to *Acinetobacter chinensis* in the Motherwell canal and sites close to inflow entry points (Figure 5.6, Figure 5.7, Table 5.1). Nivens bridge is situated ~300 m downstream of the Kat canal which flows through townships and discharges stormwater into the Swartkops river (Adams *et al*., 2019), therefore, the presence of OTU104 (*Acinetobacter chinensis*) at Nivens bridge is likely to be from runoff from townships and its absence in the upper reaches of the estuary could be due to dilution as the water flowed downstream through Perseverance bridge into the estuary. *Acinetobacter* is capable of degrading hydrocarbons (Mandri & Lin, 2007) and are used in bioremediation in textile and tannery industries effluents that contain heavy metals, and can be found in industrial waste (Doughari *et al*., 2011). These types of industries are situated in the catchment close to Motherwell and Markman canals and they could be the source of *Acinetobacter* identified in the water sampled from these sites.

Potential pathogenic bacteria were also identified in the water channels and it was evident that they were discharged into the estuary (Figure 5.9). It should be noted that while >99% sequence identity to pathogenic bacteria is a good indication of the potential occurances of these pathogens in the water column, these identifications are putative and specialized species-specific testing (which was beyond the scope of this study) would need to be conducted to verify the sequence data. In addition to high nutrient levels in the Motherwell canal, a higher proportion of pathogens were discharged from this canal into the estuary which makes it a major source of pollution in Swartkops Estuary. Pathogenic bacteria such as *Yersinia* and *Bacillus* have been reported in urban waters (Numberger *et al*., 2020) and were also identified in the urban influenced estuary in this study. *Vibrio cholerae* is associated with Cyanobacteria blooms (Ryan *et al*., 2018) which corresponds to its abundance in the Chatty river which had overgrowth of Cyanobacteria (*Phormidiaceae*) (Figure 5.6). Although *Vibrio cholerae* is a global threat, some

strains are non-virulent (Menezes *et al*., 2014) and we cannot definitively conclude that the *Vibrio* species identified in our study are toxigenic. However, their presence together with other waterborne pathogens identified in the dataset suggest that there is a potential risk of emerging outbreaks. The canals and Nivens bridge had a high relative abundance of OTU106 with 99% sequence similarity to *Aeromonas enteropelogenes* which was also identified in the estuary close to the entry points (Figure 5.7, Table 5.1). *Aeromonas enteropelogenes* was isolated from human stools and is one of the rare pathogenic bacteria that produce enterotoxins and known to cause gastroenteritis (Collins *et al*., 1993; De Luca *et al.*, 2010; Fernández-Bravo *et al.*, 2020) which means it is likely that it entered the canals and Nivens bridge from untreated sewage water.

Besides pollution from raw sewage in Markman canal, bacterial communities that are associated with industrial pollution were also identified in the canal. OTU86 with sequence similarity to *Thiobacillus* was in high relative abundance in Markman canal as well as along the estuary (Figure 5.7, Table 5.1). Members of *Thiobacillus* together with those of *Aeromonas* were found in polyethylene pipe biofilms in a study by Lequette *et al*. (2019) and they are capable of degrading polyethylene materials (Restrepo-Flórez *et al.*, 2014), which could mean that these bacterial communities were either from faulty sewage pumps or from industries discharging into Markman canal. *Thiobacillus* can solubilize heavy metals and can survive in high levels of sulfur where they use sulfur compounds as energy source (Robertson & Kuenen, 2006; Hutt *et al*., 2017), and for these reasons, they can be found in industrial wastes. Species of this genus can survive in high levels of phosphates and participate in iron cycling (Martínez *et al*., 1983), as well as nitrogen cycling (Marcial *et al.,* 2008), and are also found in biofilters in wastewater treatment plants (Le Borgne & Baquerizo, 2019) which are potential sources of pollution in Markman canal. Nutrient levels were high in Markman canal (Figure 5.3) which makes it a conducive environment for the growth of *Thiobacillus*.

The Markman canal had high relative abundances of OTUs with sequence similarity to members of *Flavobacterium* such as OTU11 and OTU15 which were also present in waterways that are commonly affected by sewage water, namely Motherwell canal, Nivens, and Perseverance bridge (Figure 5.7, Table 5.1). Nivens and Perseverance bridge flow into the upper reaches of the estuary hence the high relative abundance of these OTUs in the surface waters of the estuary which decreased towards the lower reaches. The high nutrient levels in the canals, Nivens, and Perseverance bridge may have resulted in high abundances of *Flavobacterium* species which are associated with degradation of organic matter (Sangnoi *et al*., 2016). High nutrient levels promote high phytoplankton growth, which increase levels of organic matter in the estuary and attract bacterial decomposers.

The canals, Nivens and Perseverance bridge also had a high relative abundance of OTU92 with sequence similarity to *Hydrogenophaga* which was identified in the water surface of the upper reaches of the estuary and decreased in relative abundance downstream (Appendix 3; Figure S5A, Table S15). OTU74, which also had sequence similarity to *Hydrogenophaga* sp., *was* numerically abundant in Chatty river and site 2 in the estuary close to the entry point of the Chatty river and decreased in relative abundance up the estuary (Appendix 3; Figure S5A, Table S15). *Hydrogenophaga, Flavobacterium*, and *Runella* species can be found in activated sludge (Kämpfer *et al*., 2005; Ryu *et al*., 2006; Zhang *et al*., 2017) and some species of *Hydrogenophaga* have been found in water and mud (Kämpfer *et al*., 2005; Du *et al*., 2015) and have shown to degrade pollutants (Lambo & Patel, 2006). OTUs with sequence similarity to *Flavobacterium* (OTU11, OTU15, OTU3, OTU22, OTU122, OTU33), and *Runella* (OTU57) species were in high relative abundance in Nivens bridge and at the water surface of the upper estuary and decreased in relative abundance downstream (Figure 5.7, Table 5.1, Appendix 3; Figure S5B, Table S15). *Runella* can remove nutrients and have been used as a biological treatment in wastewater to remove phosphorus from bioreactors (Ryu *et al.*, 2006), this means they flourish in nutrient-rich sites.

All sampling sites in Swartkops river (SPN, BD, NB, PSB) had a high relative abundance of bacterial OTUs with sequence similarity to *Acidovorax, Caenimonas, Polynucleobacter*, and the *Limnohabitans* including OTU65 with 99.27% sequence similarity to *Limnohabitans planktonicus* which was also in high relative abundance in Markman canal (Figure 5.7, Table 5.1). Some of the *Acidovorax* strains have previously been isolated from soil and water habitats as well as clinical specimens, but their pathogenicity has not been established (Willems *et al*., 1990). They can also be found in activated sludge (Willems & Gillis, 2015) hence their presence in high relative abundance in Swartkops river in sites close to the three wastewater treatment plants. *Acidovorax* is capable of heterotrophic denitrification (Willems *et al*., 1990) and can degrade organic micropollutants (Ning *et al*., 2010). *Caenimonas* was also found in wastewater treatment systems as polyphosphate accumulating organisms (Ji *et al.*, 2019) and in soil samples (Kim *et al.*, 2012) which could be the reason for their high relative abundance in Swartkops river.

The surrounding soils may have also played a role in structuring bacterial communities in Swartkops Estuary. *Rhizobiales Incertae sedis* is commonly found in soils and can fix nitrogen (Fischer *et al.*, 2012; Le Borgne & Baquerizo, 2019). *Rhizobiales* are linked to high nutrient conditions (Liu *et al*., 2015a). OTU98, with sequence similarity to members of this bacterial taxon, was most abundant in Nivens bridge and decreased down to Perseverance bridge and the water surface of the upper reaches where Swartkops river discharges (Appendix 3; Figure S5A, Table S15). Bulmers drift and Springfontein had high relative abundances of OTU62 with 99% sequence similarity to *Ramlibacter ginsenosidimutans.* This bacterial taxon is also associated with soils (Heulin *et al.,* 2003; Lee *et al*., 2014).

With regards to Tippers creek, we cannot confidently say it discharged pollutants from the catchment into the estuary as it had high salinity which was highly likely from the ocean. As previously mentioned, it had a high relative abundance of members of *Rhodobacteraceae Flavobacteriaceae* and *Cryomorphaceae* which likely entered the creek from the ocean (Figure 5.6) and were also prevalent in the estuary. *Rhodobacteraceae* is comprised of sulfur-oxidizing

and hydrocarbon degraders which can be found in both marine and freshwater (Chang *et al.*, 2000; Melcher *et al.*, 2002; Cytryn *et al*., 2005) and their presence in Tippers creek could also be from the stormwater inflow. Bacteria such as *Tenabaculum, Polaribacter, Marinobacterium,* and Candidatus Actinomarina are halophiles (Yoon *et al.,* 2006; Ghai *et al.*, 2013; Li *et al.,* 2014; Klakegg *et al.*, 2019; Nowlan *et al.*, 2020), and OTUs with sequence similarity to the mentioned genera were identified in high relative abundance in Tippers creek and lower reaches of the estuary and decreased in relative abundance in the upper reaches. *Polaribacter* degrades carbohydrates and has peptide utilizing enzymes that help it survive in marine oligotrophic environments (Yoon *et al*., 2017).

The Chatty river, which flows through townships and is mostly contaminated by urban runoff and untreated sewage waste (Adams *et al*., 2019), had the lowest species richness and bacterial diversity and high nutrient levels which exceeded the recommended standards for water quality. This river was predominated by members of the *Phormidiaceae* family (Figure 5.6). The most abundant OTUs within the *Phormidiaceae* family had sequence similarity to *Arthrospira* (OTU83) of which one was identified at site 2 adjacent to the entry point of the inflow point of the Chatty river and decreased as it distributed to other sites of the estuary (Figure 5.7, Table 5.1). *Arthrospira* is a freshwater chlorophyll-based phototrophic bacteria (Furmaniak *et al*., 2017; Martelli *et al*., 2020; Nourmohammadi *et al.*, 2020), although it was present in high relative abundances in Chatty river despite the higher salinity levels in the river. Although there are no chl-*a* or cell-count results as evidence of a bloom event in Chatty river, the water was extremely green during sampling (it looked like pea-soup) which shows overgrowth of *Arthrospira* which likely formed a bloom resulting in reduced overall species richness (Figure 5.5). Some bacterial genera such as PeM15_ge are associated with treated wastewater (Restrepo-Flórez *et al.*, 2014) and OTU46 with sequence similarity to this bacterium was in high relative abundance in Chatty river and at the lower reaches of the estuary and decreased in relative abundance upstream (Figure 57, Table 5.1). It may have entered the Chatty river from the wastewater discharged by industries.

As expected, some of the identified bacterial communities that flowed into Swartkops Estuary from the canals and rivers are associated with urban activities. Even though a smaller proportion of bacterial OTUs was shared between the estuary and the water channels, their presence in the estuary is evidence that urban development influenced bacterial community composition and distribution patterns in Swartkops Estuary. The Motherwell canal had a greater proportion of pollutants including high nutrient concentrations and many potentially pathogenic bacteria that were also identified in the estuary. This indicates that while the artificial wetland significantly reduced harmful input into the estuary, it is overloaded and anthropogenic inputs into the estuary from the Motherwell canal is still occurring. Perseverance and Nivens bridge were the most polluted sites sampled from within the river and had a greater influence on the bacterial structure in the upper estuary compared to that of Bulmers drift and Springfontein. Nivens and Perseverance bridge discharged bacterial OTUs, of which some are potentially pathogenic and non-fecal, into the estuary while Tippers creek contained bacterial communities which mostly survive in high salinity environments. Overall, the water inflow from the sampled water channels discharged pollutants into Swartkops Estuary and influenced its bacterial structure.

CHAPTER 6: CONCLUSIONS

Anthropogenic activities contribute immensely to the poor water quality and ecosystem functioning in estuaries (Nedwell *et al.*, 2002; Woodland *et al*., 2015; Tian *et al.,* 2020). The two major causes of water deterioration, namely agricultural and urbanized/industrial activities, influenced microbial community composition and diversity in the Sundays and Swartkops Estuaries. Agricultural activities have emerged as significant contributors to estuarine pollution in many studies (Hapeman *et al.*, 2002; Ramos e Silva *et al.*, 2017; Lemley *et al.,* 2018b; Conrad *et al.,* 2019; Adams *et al.,* 2020; Dudley *et al.,* 2020) and the impacts of agriculture in the catchment area were evident in Sundays Estuary. Urban activities have also shown to be problematic by discharging pollutants in estuaries through stormwater discharge (Basnyat *et al.,* 1999; Lapointe *et al.*, 2012; Fischer *et al.*, 2015; Levin *et al*.,2020). Stormwater discharge as well as poor management/spillage from wastewater treatment plants were observed to impact the Swartkops Estuary in this study.

Pollution from agricultural and urbanised activities was apparent in both the estuaries in this study with increased levels of nutrients, and the presence and abundance of bacterial and eukaryotic communities associated with pollutants in the estuaries. High nutrient levels entered the estuaries from the upper reaches where agricultural farming is practiced in the catchment of Sundays Estuary and where there are three poorly maintained sewage treatment plants in the catchment of Swartkops Estuary. Since urban estuaries are commonly contaminated from point sources (McCarthy *et al.*, 2017), further assessment on stormwater inflow from canals, rivers, and creek to identify sources of pollution in Swartkops Estuary was carried out. According to the results, stormwater had high nutrient levels which were discharged at different sites along the length of Swartkops Estuary. Although rivers are usually not referred to as stormwater sources, they contribute to stormwater input in estuaries (Jovanovic *et al*., 2015), as is the case with Swartkops river which receives stormwater from the Kat canal which passes through townships, and the Chatty river which runs through highly populated residential areas (Adams *et al.,*

2019). The high levels of nutrients in Sundays and Swartkops Estuaries in this study concur with previous studies that showed nutrient inputs as the main cause of pollution (Adams *et al*., 2019; Lemley *et al.*, 2020).

Phosphates and TOxN, which are considered the major causes of estuarine pollution (Nedwell *et al.*, 2002; Silva *et al.,* 2015; Woodland *et al*., 2015), were in excessive amounts in both estuaries with higher TOxN concentrations in Sundays Estuary while Swartkops Estuary had overall high levels of phosphates. The differences in the type and concentrations of nutrients in the estuaries were influenced by the activities taking place in the catchment. Whilst there is extensive agricultural farming in the upper reaches of Sundays Estuary, Swartkops Estuary receives stormwater from urbanized and residential areas. The high concentration of TOxN is indicative of fertilizer application which was probably from agricultural return flow in Sundays Estuary. Phosphates are indicative of anthropogenic inputs which may enter the estuary from urbanised and agricultural activities. Swartkops Estuary has limited agricultural activities taking place in the catchment, which means the bulk of pollutants were from the residences and industries. High levels of phosphates mostly occur in urban estuaries as a result of sewage input (Silva *et al*., 2015) which is true for Swartkops Estuary. Wastewater can also be a source of nitrogen in aquatic systems (Valiela & Bowen, 2002). The stormwater inflow from the canals associated with the Swartkops Estuary are regularly affected by raw sewage water (Adams *et al*., 2019) and had higher concentrations of TOxN than phosphates while the Perseverance bridge sampling site which is also situated close to the sewage treatment plants had higher phosphate levels. These water channels had a high concentration of ammonium which was most likely from the sewage waste.

Even though Sundays Estuary had lower concentrations of phosphates than the Swartkops Estuary, the phosphorus and nitrogen levels exceeded the recommended threshold in both estuaries which means they were both a health risk to the inhabitants and surrounding human

communities. Anthropogenic nutrients degrade water quality (Zheng *et al.,* 2019) by altering biological community composition and function within estuaries (Woodland *et al*., 2015). Nitrogen and phosphorus are utilized in bacterial metabolism and phytoplankton growth, but negatively impact the estuarine systems when in high concentrations by promoting algal blooms (reviewed by Aryal *et al.*, 2010). The nutrient data from Swartkops shows that the estuary was polluted in all seasons and caused *Bacillariophyceae* blooms during sampling in summer and autumn. A *Bacillariophyceae* (*Cyclotella*) bloom was also noted during sampling in Sundays Estuary in 2019 while *H. akashiwo* formed the bloom in 2018. Both estuaries hold a history of bloom events (Scharler & Baird, 2003; Kotsedi *et al.,* 2012; Lemley *et al*., 2017, 2020) which is also reflected in the results of this study, particularly in Sundays Estuary. Eutrophication in Sundays Estuary was indicated by the presence and abundance of *Stephanodiscus hantzschii* and *Scenedesmus* which grow best in nutrient-rich environments. Some of the dominant phytoplankton communities include *Dinophyceae* which were in high relative abundance in Sundays Estuary during sampling in 2018 when nutrient levels were higher and bloomed during spring sampling in Swartkops Estuary.

The inputs from anthropogenic-derived sources contribute to fluctuations in physico-chemical conditions (Courtenay *et al.*, 2011) which impacts the bacterial diversity and composition in estuaries (Crump *et al.*, 2007). Blooms caused by anthropogenic nutrients correlated with increased pH and temperatures in Swartkops Estuary and dissolved oxygen in the two estuaries which was likely a result of photosynthetic activity. According to the phytoplankton results, high cell counts were identified in the surface water due to exposure to warmer temperatures and sunlight for photosynthesis. Since samples were only collected during the spring season in Sundays Estuary, we cannot be certain about the influence of temperature on algal blooms as it was relatively consistent along the length of the estuary during sampling in the two years. It was clear that anthropogenic nutrients played a major role in algal blooms.

Different estuaries harbor different bacterial communities depending on the ecological adaptation of bacterial communities (Jinjun & Jun, 2012). The two estuaries had a high abundance of members of the phylum Proteobacteria followed by the Bacteroidetes. Although this study focused on bacterial communities in the water column where Proteobacteria were identified in abundance, the same bacterial community is common in estuarine systems and was abundant in the estuarine sediments (Yi *et al*.,2020). Most of the dominant bacterial communities in Swartkops Estuary were of the class Alphaproteobacteria, which were mostly halophiles, namely SARII_clade *(*Clade_I, Clade_II*),* SAR116_clade, and *Rhodobacteraceae*. These bacterial communities were found in high relative abundance along the length of the estuary with the exception of the water surface of the upper reaches during sampling in autumn and winter and upper reaches during sampling in summer. The same marine bacterial taxa were dominant in Sundays Estuary, but lower in abundance compared to Swartkops Estuary.

Bacterial communities identified in the Sundays and Swartkops Estuaries were mostly heterotrophic, with few taxa of autotrophic bacteria. Within the bacterial dataset from Sundays Estuary, *Nostocaceae* was the only dominant autotrophic bacterial taxon that was identified in high relative abundance at the upper reaches during the 2019 sampling, while *Cyanobiaceae* and *Oxyphotobacteria* were high in relative abundance at the upper reaches of Swartkops Estuary during sampling in summer and winter seasons, respectively. Some of the heterotrophic bacteria observed in high relative abundance in bloom sites, particularly in Sundays Estuary, were those that have been associated with phytoplankton (Buchan *et al*., 2014; D'Ambrosio *et al*., 2014; Teeling *et al*., 2016). Even though *Flavobacteriaceae* can be influenced by salinity (Zhang *al.,* 2014), they are associated with phytoplankton growth (Pinhassi *et al*., 2004) and were abundant in the estuaries. *Cryomorphaceae, Halieaceae*, and OTUs with sequence similarity to *Rheinheimera* showed a close association with *H. akashiwo* blooms during sampling in 2018 while *Rubritaleaceae,* which are also associated with phytoplankton, were abundant at the upper reaches of Swartkops Estuary during *Bacillariophyceae* blooms in summer. Since the Sundays Estuary had high anthropogenic nutrient levels at the upper reaches, some of the bacterial

communities that were identified in abundance were those that can utilize nitrates such as NS11-12_marine group *w*hich was in low relative abundance during sampling in Swartkops Estuary. The abundance of *Sporichthyaceae* and *Erysipelotrichaceae* in the upper reaches of Sundays Estuary were indicative of agricultural inputs as these bacterial communities are mostly associated with compost manure (Weglarz *et al*., 2018), while they were present but non-dominant in Swartkops Estuary. Bacterial OTUs, such as OTU39 with sequence similarity to *Polynucleobacter duraqueae*, have been shown to be associated with high phosphorus content and humic compounds (Hahn *et al*., 2016) and were identified at the upper reaches of Swartkops Estuary where there is a frequent inflow of untreated sewage waste. Although bacterial communities in the two estuaries were impacted by different anthropogenic activities, they both had high relative abundances of *Burkholderiaceae* in the upper reaches with *Hydrogenophaga* as one of the abundant genera. Members of *Burkholderiaceae* were abundant on the water surface during sampling in autumn and winter in Swartkops Estuary, which was likely due to freshwater influx which may have flushed organic matter into the estuary or decayed phytoplankton that was already in the estuary. *Burkholderiaceae* were also high in relative abundance during sampling in 2018 when Sundays Estuary had high nutrient levels. Some of the industrially and agriculturaly impacted estuaries include the Pearl River Estuary in China in which bacterial distribution patterns were defined by nutrient levels as with the Sundays and Swartkops estuaries (Zhang *et al*., 2014).

Urbanised estuaries are commonly polluted by pathogenic bacteria as with Swartkops and other estuaries such as the Karnaphuli Estuary studied by Kopprio *et al*. (2020). In a study by Kopprio *et al.* (2020), Karnaphuli Estuary in Bangladesh was contaminated by sewage waste and some bacterial communities which were identified were similar to the ones identified in Swartkops Estuary, such as *Acinetobacter, Arcobacter* and *Aeromonas.* The same estuary also had strong estuarine gradient which likely influenced the presence of Rhodobacterales, Oceanospirillales and Cellvibrionales which were also identified in the Swartkops Estuary with a strong salinity gradient. According to the data generated in this study, sampling sites in the Swartkops river (SPN, BD, NB, PSB) and the canals were the major sources of sewage waste. This was indicated by high abundances of *Rhodocyclaceae, Arcobacteriaceae*, and *Aeromonadaceae* which were

observed in the point input water sources into the Swartkops Estuary. While these bacterial families were present in the Sundays Estuary, they were non-dominant. Pipes used in industries and wastewater treatment plants are a niche for some bacterial communities (Fisher *et al.*, 2015) such as those that entered the estuary through the canals including OTU104 with sequence similarity to *Acinetobacte*r and OTU86 with sequence similarity to *Thiobacillus.*

The Motherwell canal had the highest nutrient levels compared to other water channels flowing into the Swartkops Estuary and carried most potentially pathogenic bacteria including *Aeromonas caviae* (*Aeromonadaceae*)*, Shigella sonnei*, and *Yersinia enterolitica* which are of the *Enterobacteriaceae* family. The Nivens bridge sampling site in the Swartkops river also had a high abundance of *Aeromonas caviae* which is a human pathogen that can be found in aquatic systems (Altwegg, 1985; Batra *et al.,* 2016). *Shigella sonnei* has been identified as one of the pathogenic bacteria in many industrialised regions (Thompson *et al*., 2015; Torraca *et al.,* 2020) and it is common in low, and middle-income countries (Shad & Shad, 2021) which corresponds to the findings from the Swartkops in this study. As with the Motherwell canal, the Chatty river and Markman canal had high nutrient levels which caused *Arthrospira* sp. blooms in Chatty river and likely resulted in low bacterial richness. The Chatty river had a high abundance of *Vibrio cholerae* which is also a potentially pathogenic microorganism. *Vibrio cholerae* is a natural inhabitant of riverine and estuarine systems (Kierek & Watnick, 2003) that can cause cholera outbreaks (Dalusi *et al*., 2015) and can be found in environments rich in nitrogen and phosphorus (Lutz *et al*., 2013) which was the case with the Chatty river. Most of the bacterial biomass that entered Swartkops Estuary through canals, rivers, and creek were not a risk to human health, nonetheless, they play a role in the ecosystem functioning and contributed to microbial composition and diversity in the estuary.

Besides anthropogenic activities, environmental variables play a role in mapping distribution patterns of bacterial communities in the estuaries. Swartkops Estuary had a stronger salinity gradient at the time of sampling than Sundays Estuary, which was also seen in the previous

results in a study by Scharler & Baird (2003). This could be due to catchment runoff as well as from the inter-basin transfer scheme in Sundays Estuary while Swartkops Estuary gets input from runoff in the catchment area and canals which were very shallow at the time of sampling, and possibly discharged very low amounts of freshwater. Despite the differences in salinity gradient between the Sundays and Swartkops estuaries, statistical analysis (Spearman rank correlation) showed salinity to be the major driver of biological variation in both estuaries as was also found in a study by Crump *et al*. (2004) and in the northern hemisphere, in a study by Zhang *et al.* (2014). The main drivers of phytoplankton distribution patterns in a study by Barrosso *et al.* (2016) were salinity and total nitrogen from freshwater inflow which also impacted the phytoplankton structure in both Sundays and Swartkops Estuaries. In addition to salinity, all other environmental variables that were measured in the estuaries played a role in microbial compositional variation, with chl-*a* as the least contributing factor.

Anthropogenic inputs and environmental variables also influenced the distribution patterns of animals and fungi that were identified in the 18S rRNA metabarcoding dataset from Sundays Estuary. The Arthropoda were identified in abundance at the upper estuary during sampling in 2019 while most of the members of the phylum Ciliophora that were identified in 2018, were those that can survive in high salinities. Different Arthropoda respond differently to environmental factors (David *et al.,* 2016), and with the dataset generated in this study, some families survived in high saline waters like *Oithona* sp. (OTU49) while others preferred sites at the upper reaches of the estuary where there was an overgrowth of reeds and high nutrient levels such as OTU116 (sequence similarity to *Aculodes* sp.). Fungi were only in high abundance during sampling in 2019 at the upper reaches in low salinity and nutrient-rich sites. Due to time constraints and restricted access to the laboratory due to the covid pandemic, the 18S rRNA metabarcoding of the Swartkops Estuary could not be completed and was thus not reported on in this thesis.

In terms of future research and monitoring in South African estuarine ecosystems, anthropogenic pollution in the Sundays and Swartkops Estuaries is of concern and needs to be monitored on a regular basis with feedback given to the relevant authorities in order for suitable mitigating actions to be implemented. The occurrence of other pollutants, such as heavy metals and chemicals, within the Sundays and Swartkops Estuaries were beyond the scope of this study. However, these pollutants can significantly influence biological community structure and diversity in estuarine systems (Hu *et al.,* 2012; Abdulaziz *et al*., 2017; Zhang *et al.,* 2020b), and assessing their levels in the Sundays and Swartkops Estuaries would be of benefit in understanding the complexity of ecosystem functioning and anthropogenic impacts. This study focused solely on the composition of the microbial community within the water columns of the target estuaries and assessment of the benthic microbial community would be of great interest because many pollutants settle and are concentrated within the sediments (Forsberg, 1989) and thus benthic microbiota may well be a better indicator of long-term, persistent, and degradation-resistant pollution events.

The presence of potentially pathogenic bacteria in all seasons in the Swartkops Estuary is highly concerning. These potentially pathogenic bacteria identified in the water channels and Swartkops Estuary need to be studied further to establish their pathogenicity, the community educated to avoid disease outbreaks, and governing bodies informed and held to account with respect to proper management of wastewater treatment plant and implementation of measure to address the poor water quality of these estuaries. Although stormwater from the Motherwell canal passes through an artificial wetland before flowing into the estuary, it is not completely effective as it carried high levels of nutrients and more potentially pathogenic bacteria than other water channels. This is most likely due to over-burdening of the artificial wetland with water inflow exceeding its remediation capacity (pers. Comm. J. Adams). Expansion and regular maintenance of this artificial wetland would likely improve this point source inflow into the estuary and should be routinely monitored.

To date, reports in the literature on pollution of Swartkops and Sundays Estuaries have been based on physico-chemical data and morphological assessment of phytoplankton growth (Lemley *et al*., 2017, 2018b, 2020; Scharler & Baird, 2003; Adams *et al.,* 2019). This has been significantly expanded upon by the genetic analyses, via DNA metabarcoding, of the microbial community found in these estuaries and reported on in this study.

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APPENDIX 1: Supplementary data for Chapter 3

Table S1: *Physico-chemical parameters measured through the water column of Sundays Estuary in 2018 (A) and 2019 (B)*

Sites (2018)	Distance from mouth (km)	Depth (m)	pH	Temperature (°C)	Salinity (ppt)	DO (mg/l)
Site 1	2.6	0	8.31	20.40	25.20	6.91
		0.5	8.30	19.70	26.60	6.91
		1	8.30	19.60	27.80	7.05
		1.324	8.30	19.20	28.19	7.14
Site 2	4.5	0	8.39	21.80	20.89	6.90
		0.5	8.38	21.60	21.01	6.86
		1	8.35	20.90	23.24	6.75
Site 3	8.6	0	8.54	23.10	13.72	8.27
		0.5	8.52	22.70	13.94	8.05
		1	8.49	22.40	14.81	7.44
		1.5	8.44	22.00	16.36	6.65
		2	8.39	21.80	17.82	6.02
		2.5	8.36	21.50	18.74	5.50
Site 4	10.5	0	8.66	23.50	9.06	12.82
		0.5	8.66	23.50	9.07	13.81
		1	8.59	22.70	9.88	10.81
		1.5	8.50	22.30	12.98	7.71
Site 5	13.8	0	8.66	23.40	3.43	12.21
		0.5	8.66	23.50	3.47	13.45
		1	8.60	23.00	3.72	12.31
		1.5	8.51	22.30	5.59	8.15
Site 6	17.6	0	8.40	22.60	2.27	6.91
		0.5	8.37	22.60	2.28	6.79
		1	8.36	22.40	2.29	6.50
Site 7	20.1	0	8.35	22.30	2.28	6.60
		0.5	8.34	22.20	2.28	6.54
		1	8.33	22.10	2.29	6.47
		1.5	8.33	22.20	2.29	6.47
Site 8	21.6	0	8.37	22.20	2.38	7.11
		0.5	8.36	22.10	2.38	7.05
		1	8.36	22.10	2.38	7.05
		1.5	8.36	22.10	2.38	7.02
		2	8.35	22.10	2.38	7.01

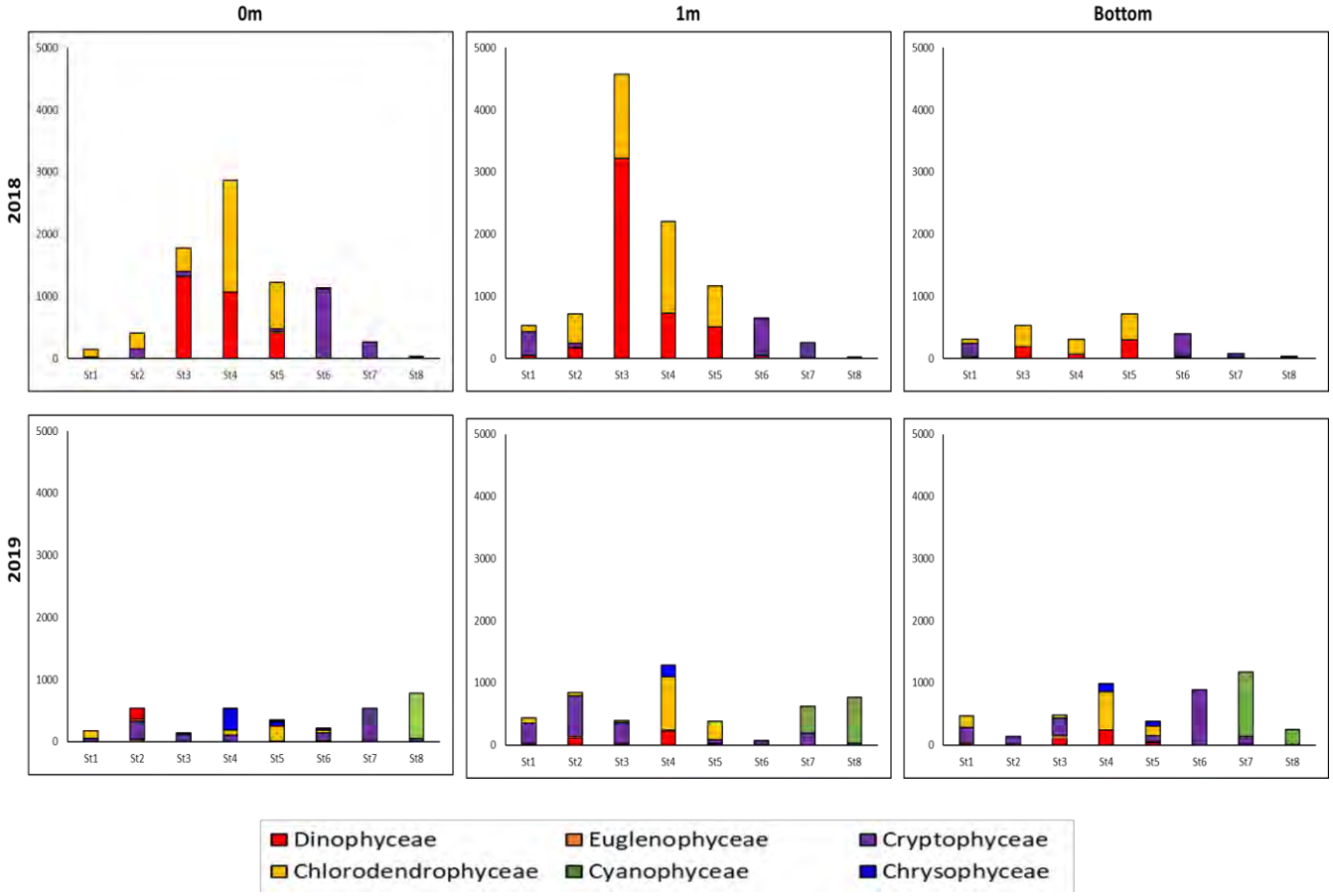

Figure S1: *Non-dominant phytoplankton biomass through the water column of Sundays Estuary in 2018 and 2019 spring sampling*

Table S2*: Statistical results from ANOSIM analysis of eukaryotes OTUs generated from the water column of Sundays Estuary against selected variables. Values were calculated using Bray Curtis distance matrix.*

	Groups	R Statistic	p-value	Permutations
2018	Global	0.776	0.001	999
	Polyhaline, Mesohaline	0.749	0.1	999
	Polyhaline, Oligohaline	0.909	0.1	999
	Mesohaline, Oligohaline	0.79	0.1	999
2019	Global	0.819	0.001	999
	Polyhaline, Marine	0.921	0.001	999
	Polyhaline, Mesohaline	0.401	0.001	999
	Polyhaline, Oligohaline	1	0.001	999
	Marine, Mesohaline	0.997	0.002	999
	Marine, Oligohaline	1	0.001	999
	Mesohaline, Oligohaline	0.997	0.001	999

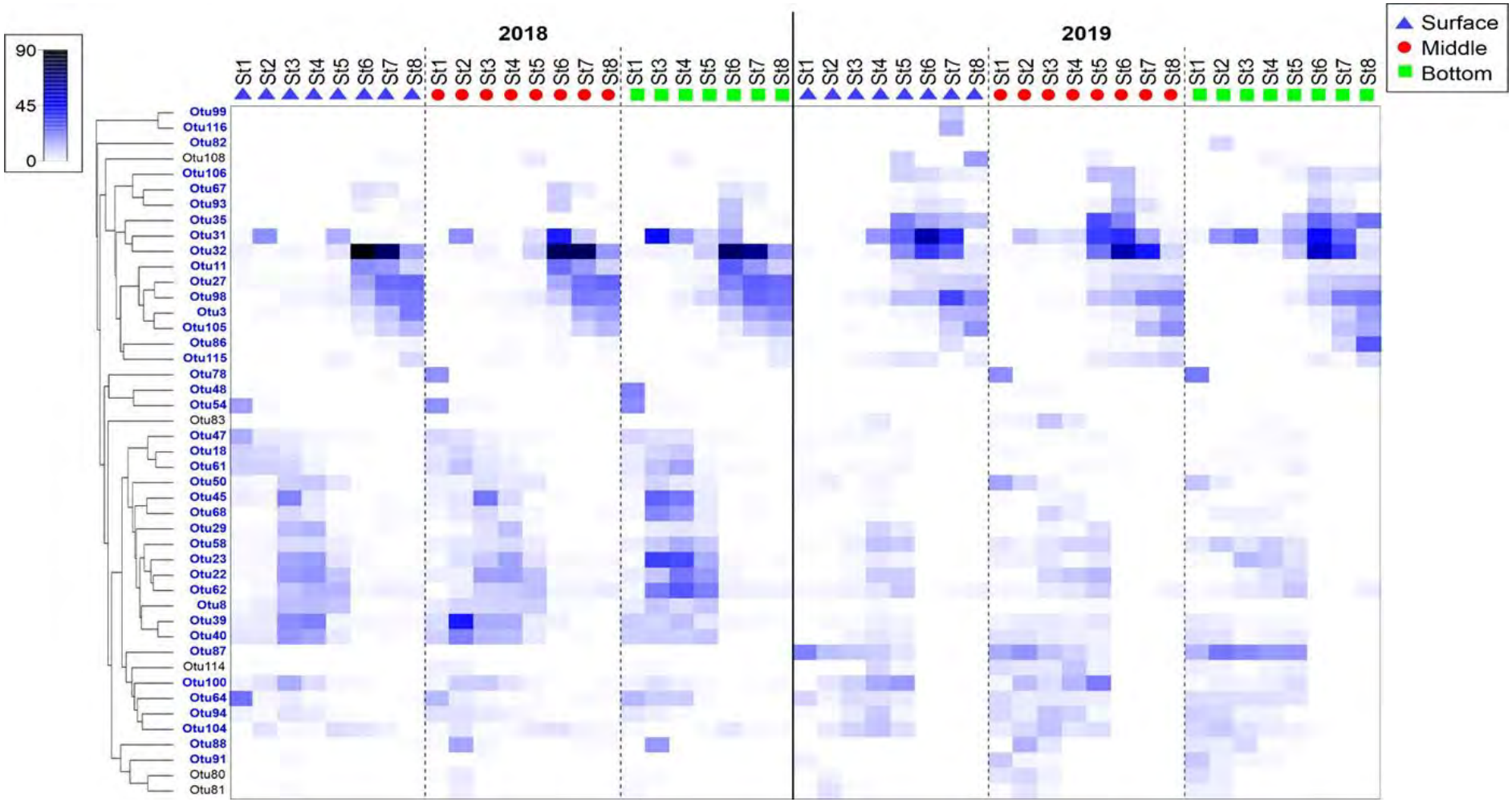

Figure S2.A: Heatmap of the top most dominant OTUs of the unclassified eukaryotes sampled through the water column of Sundays Estuary in 2018 and 2019. OTUs were generated at a distance of 0.03. OTUs of interest are highlighted in blue color. Lower reaches of the estuary (sites 1, 2), Middle reaches (sites 3, 4, 5), Upper reaches (sites 6, 7, 8).

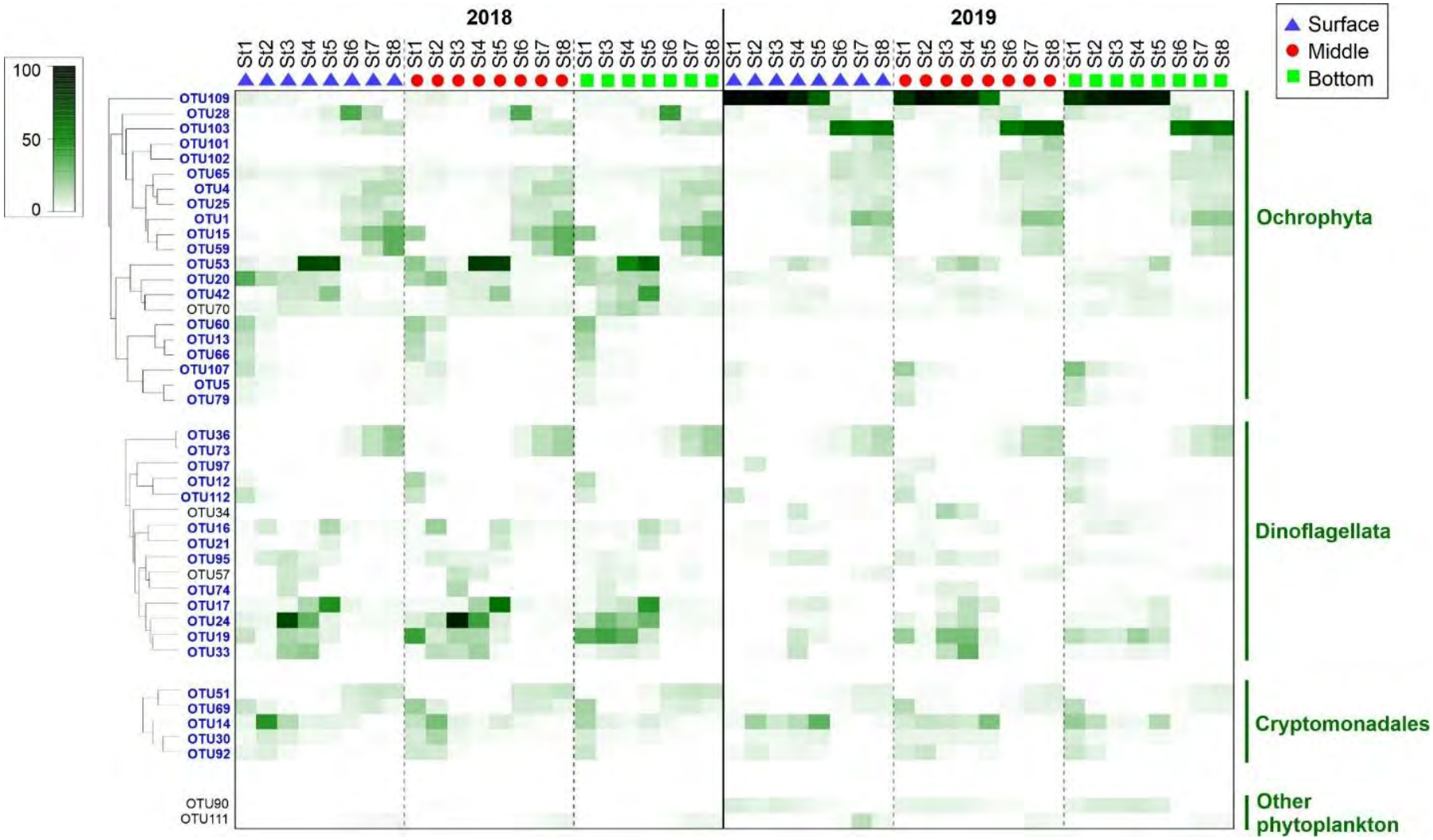

Figure S2.B: Heatmap of the most dominant phytoplankton OTUs of the phyla Ochrophyta, Dinoflagellata, Cryptomonadales and 'Other phytoplankton' (MAST-6 and Phragmoplastophyta) sampled through the water column of Sundays Estuary in 2018 and 2019. OTUs were generated at a distance of 0.03. OTUs of interest are highlighted in blue color**.**

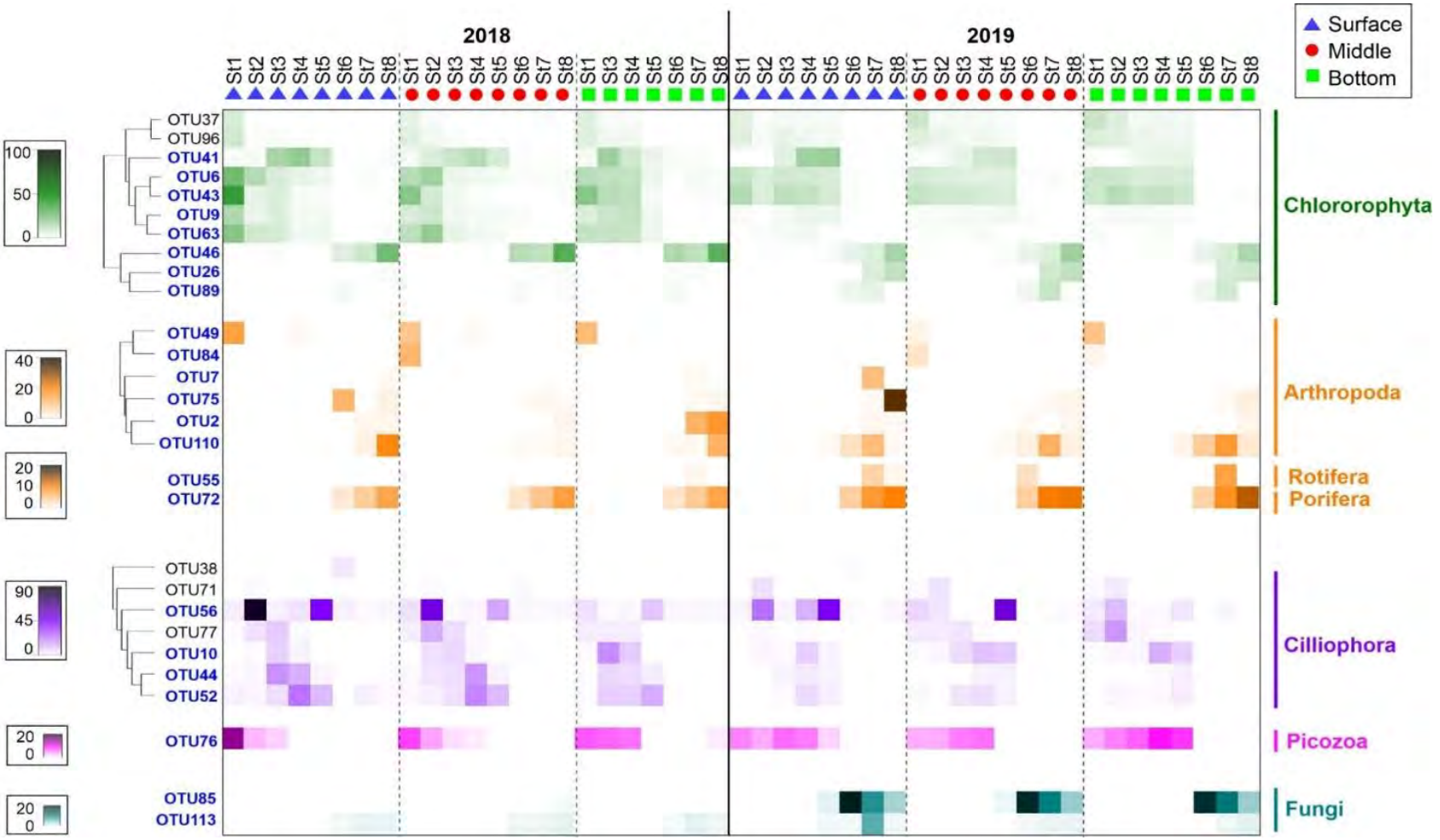

Figure S2.C: Heatmap of the top most dominant eukaryotes OTUs of the phyla Chlorophyta, Arthropoda, Rotifera, Porifera, Cilliophora, Picozoa and Fungi, sampled through the water column of Sundays Estuary in 2018 and 2019. (n=2). OTUs were generated at a distance of 0.03. OTUs of interest are highlighted in blue color

Table S3: *Blast match results of eukaryotes OTUs at genus/species level against the GenBank database and Silva_v132 reference database*

OTUs	**Closest Match against Silva v132**	**Match against nr/nt GenBank**		
		Accession Number	**Identity match %**	**Closest Taxonomic match in NCBI**
OTU1	*Bacillariophytina*_unclassified	KU948210.1	95.65%	*Cocconeis placentula*
OTU3	Eukaryota_unclassified	JF791059.1	89.94%	*Thalassiosira minima*
OTU4	*Bacillariophyceae*_unclassified	KJ961668.1	98.20%	*Navicula arenaria*
OTU5	*Diatomea*_unclassified	KJ758316.1/ KJ758234.1/ KJ757812.1	94.61%	uncultured eukaryote
OTU6	*Chlrophyta*_ph_unclassified	FR874798.1	98.21%	uncultured marine picoeukaryote
OTU7	Arthropoda_unclassified	KY230784.1	98.82%	*Stenacidia violacea*
OTU8	Eukaryota_unclassified	FN562440.1	98.25%	*Pyramimonas disomata*
OTU9	*Chlorophyta*_ph_unclassified	HE610136.1	94.97%	*Marsupiomonas pelliculata*
OTU10	*Haptoria*_unclassified	KY887579.1	100%	*Monodinium* sp.
OTU11	Eukaryota_unclassified	KP768127.1	93.10%	*Sardina pilchardus*
OTU13	*Dictyocha*	KJ763206.1	99.40%	uncultured eukaryote
OTU14	*Cryptomonadales*_ph_unclassified	FJ884690.1	100%	*Cryptophyta* sp.
OTU15	*Mediophyceae*_unclassified	DQ093370.1	100%	*Stephanodiscus hantzschii*
OTU16	*Dinophyceae*_unclassified	KJ760061.1	98.18%	uncultured eukaryote
OTU17	*Dinophyceae*_unclassified	KC336918.1	97.56%	uncultured eukaryote
OTU19	*Dinophyceae*_unclassified	FN669510.1	97.01%	*Gyrodinium dominans*
OTU20	*Ochrophyta*_unclassified	EF432518.1	98.20%	*Paraphysomonas imperforata*
OTU21	*Dinoflagellata*_unclassified	AY033487.1	99.40%	*Pfiesteria*-like dinoflagellate sp.
OTU22	Eukaryota_unclassified	MW139914.1	98.83%	uncultured eukaryote
OTU23	Eukaryota_unclassified	EF527134.1	100.00%	marine eukaryote
OTU24	*Suessiaceae*_unclassified	KJ763207.1	100%	uncultured eukaryote
OTU25	*Mediophyceae*_unclassified	JN090903.1	100%	uncultured eukaryote
OTU26	*Chlorophyceae*_unclassified	KJ413176.1	99.40%	*Scenedesmus* sp.
OTU27	Eukaryota_unclassified	KJ961656.1	92.77%	*Surirella* sp.
OTU28	*Bacillariophytina*_unclassified	KT347147.1	97.60%	*Conticribra weissflogiopsis*

OTU29	Eukaryota_unclassified	FJ480419.1	98.14%	Strombidium basimorphum
OTU30	*Hemiselmis*	JQ420121.1	98.05%	phytoplankton
OTU31	Eukaryota_unclassified	KP768155.1	92.26%	*Pseudodiaptomus marinus*
OTU32	Eukaryota_unclassified	FR874777.1	98.19%	marine picoeukaryote
OTU33	*Dinophyceae*_unclassified	KJ764350.1/ KJ763333.1/ KJ763322.1/	94.01%	uncultured eukaryote
OTU35	Eukaryota_unclassified	JF781537.1	95.86%	*Cyclopina gracilis*
OTU36	Dinoflagellata_unclassified	L19069.1	92.68%	*Cryptosporidium muris*
OTU39	Eukaryota_unclassified	AB639343.1	91.62%	*Amphidiniopsis rotundata*
OTU40	Eukaryota_unclassified	JN048125.1	98.83%	uncultured eukaryote
OTU41	*Prasinophytae*_unclassified	KC336982.1/ KC336977.1/ KC336961.1	100%	Uncultured Eukaryote
OTU42	*Bacillariophytina*_unclassified	KC771201.1/ KC771150.1	97.59%	uncultured marine eukaryote
OTU43	*Mamiellales*_unclassified	FR874768.1	100%	uncultured marine picoeukaryote
OTU44	*Oligotrichia*_unclassified	FJ480419.1	96.27%	*Strombidium basimorphum*
OTU45	Eukaryota_unclassified	FR874831.1/ FR874805.1/ FR874799.1	100%	uncultured marine picoeukaryote
OTU46	*Ulvales*_fa_unclassified	MN070070.1/ MN070069.1	98.22%	*Ulva flexuosa*
OTU47	Eukaryota_unclassified	AY625894.1	98.17%	*Chaetoceros calcitrans f. pumilus*
OTU48	Eukaryota_unclassified	LT631075.1	100%	*Acropora granulosa*
OTU49	*Cyclopoida*_ge	KX364946.1	100%	*Oithona* sp.
OTU50	Eukaryota_unclassified	FN562440.1	99.42%	*Pyramimonas disomata*
OTU51	*Cryptomonadales*_fa_unclassified	AJ007277.1	99.40%	*Chroomonas* sp.
OTU52	*Oligotrichia*_unclassified	EF486862.1	98.14%	*Omegastrombidium elegans*
OTU53	*Heterosigma*	AY788934.1	100%	*Heterosigma akashiwo*
OTU54	Eukaryota_unclassified	FR874449.1	90.70%	uncultured marine picoeukaryote
OTU55	*Ploimida*_ge	MK271750.1	100%	*Brachionus calyciflorus*
OTU56	*Oligotrichia*_unclassified	EF100412.1	100%	*uncultured eukaryote*

OTU57	*Dinophyceae*_unclassified	EU371181.1	93.45%	uncultured marine eukaryote
OTU58	Eukaryota_unclassified	KC771146.1	98.18%	uncultured marine eukaryote
OTU59	*Mediophyceae*_unclassified	EF585582.1	98.80%	*Conticribra weissflogii*
OTU60	*Diatomea*_unclassified	KT347147.1	98.8	*Conticribra weissflogiopsis*
		DQ093368.1		*Thalassiosira tumida*
OTU62	Eukaryota_unclassified	AY180032.1	100%	uncultured eukaryote
OTU63	Chlorophyta_ph_unclassified	X75565.1	97.04%	*Pseudoscourfieldia marina*
OTU64	Eukaryota_unclassified	KT878714.1/ KT860601.1/ KT860600.1/	100%	*Bathycoccus prasinos*
OTU65	*Diatomea*_unclassified	JN090893.1	98.20%	uncultured eukaryote
		EU143937.1		uncultured stramenopile
		DQ093367.1/ AF374481.2		*Thalassiosira pseudonana*
OTU66	*Diatomea*_unclassified	KC894153.1/ KC894152.1/ KC814812.1	98.79%	*Tenuicylindrus belgicus*
OTU67	Eukaryota_unclassified	FR874777.1	97.59%	uncultured marine picoeukaryote
OTU68	Eukaryota_unclassified	KU757391.1	88.34%	uncultured eukaryote
OTU69	*Teleaulax*	MH107135.1	100%	*Teleaulax amphioxeia*
OTU72	*Spongillida*_ge	KC899073.1	100%	*Ephydatia fluviatilis*
OTU73	*Dinophyceae*_unclassified	KJ760061.1/ KJ759623.1/ KJ757402.1/	92.12%	uncultured eukaryote
OTU75	Maxillopoda_unclassified	GU070895.1/ GU070874.1/ GU070866.1	100%	invertebrate environmental
OTU76	*Picomonas*	JX988758.1	98.24%	*Picomonas judraskeda*
OTU78	Eukaryota_unclassified	KJ760838.1	98.19%	uncultured eukaryote
OTU79	*Bacillariophyceae*_unclassified	AY216904.1/ X77701.1	99.40%	*Asterionellopsis glacialis*
OTU82	Eukaryota_unclassified	JF781544.1	94.61%	*Lichomolgus marginatus*
OTU84	Arthropoda_unclassified	EU371205.1	100%	uncultured marine eukaryote

OTU85	*Kappamyceataceae*_ge	EU162640.1	99.38%	uncultured *Chytridiomycota*
OTU86	Eukaryota_unclassified	AB076630.1	92.90%	*Cytheromorpha acupunctata*
OTU87	Eukaryota_unclassified	MK021829.1	100%	uncultured eukaryote
OTU89	*Chlamydomonadales*_fa_unclassified	AF252547.1	98.80%	*Wislouchiella planctonica*
OTU91	Eukaryota_unclassified	KF130083.1	97.96%	uncultured eukaryote
OTU92	*Cryptomonadales*_fa_unclassified	JQ420121.1	98.05%	phytoplankton
OTU93	Eukaryota_unclassified	JX012185.1		*Strombidium stylifer*
		FJ543107.1	95.68%	*Strombidium* sp.
OTU94	Eukaryota_unclassified	KX611141.1	95.91%	*Pyramimonas parkeae*
OTU95	*Dinophyceae*_unclassified	AY179993.1	89.35%	uncultured stramenopile
OTU98	Eukaryota_unclassified	AY179993.1	89.35%	uncultured stramenopile
OTU99	Eukaryota_unclassified	MF077708.1	96.71%	*Halicyclops* sp.
OTU101	*Ochrophyta*_unclassified	AY642717.1	100%	eukaryotic picoplankton
OTU102	*Mediophyceae*_unclassified	KT346253.1	98.80%	uncultured eukaryote
OTU103	*Cyclotella*	KY364697.1	98.20%	*Cyclotella cryptica*
OTU104	Eukaryota_unclassified	FR874777.1	100%	uncultured marine picoeukaryote
OTU105	Eukaryota_unclassified	KJ759486.1	94.01%	uncultured eukaryote
OTU106	Eukaryota_unclassified	JF781537.1		*Cyclopina gracilis*
		JF781536.1	95.27%	*Notodelphys prasina*
OTU107	*Minidiscus*	DQ093363.1	98.80%	*Minidiscus trioculatus*
OTU108	Eukaryota_unclassified	AY627002.1	94.01%	*Anthessius* sp.
OTU109	*Cyclotella*	KT346253.1	98.80%	uncultured eukaryote
OTU110	Arthropoda_unclassified	EU380307.1	91.57%	*Attheyella crassa*
OTU113	Peronosporomycetes_fa_unclassified	MN306220.1/ MN306217.1	97.63%	*Albugo candida*
OTU115	Eukaryota_unclassified	MK302474.1	97.04%	*Heterolepidoderma sinus*
OTU116	Eukaryota_unclassified	KJ841948.1	98.01%	*Aculodes* sp.

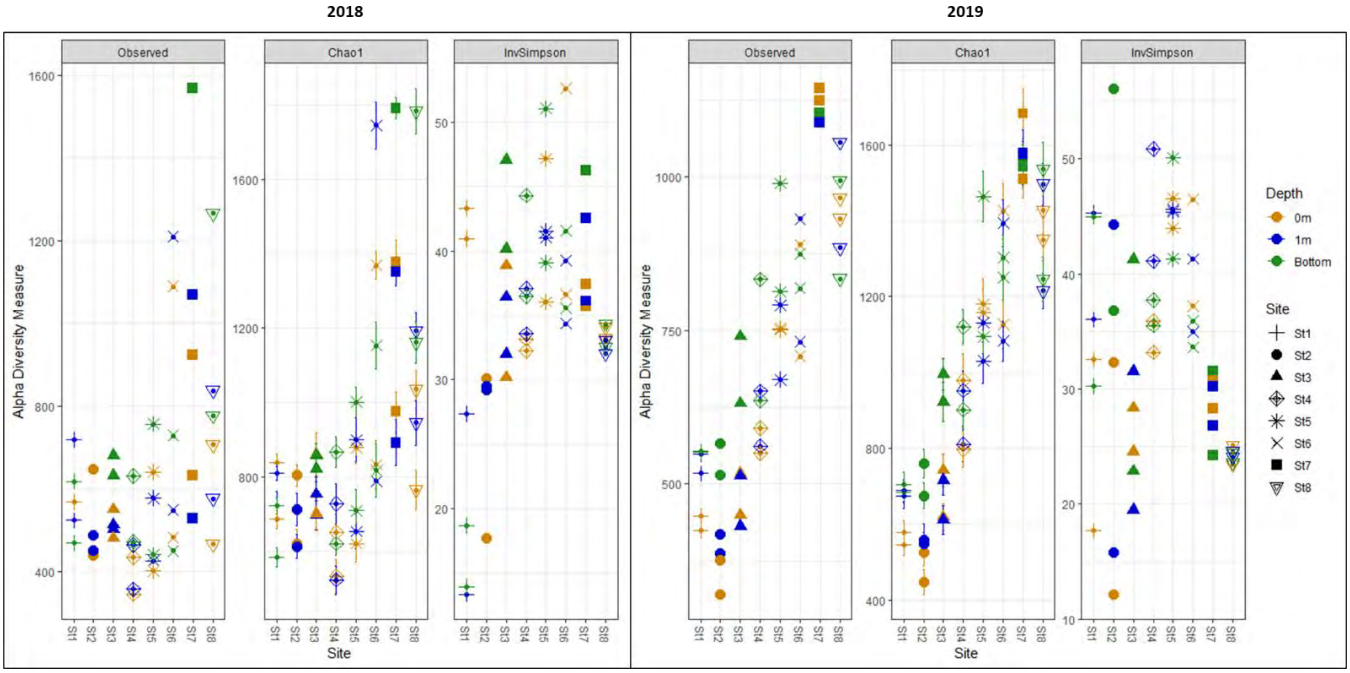

***Figure S3**: Alpha diversity indices representing species richness (Observed and Chao1) and species richness and evenness (InvSimpson) of the unsubsampled bacterial OTUs in the surface (0m), middle (1m) and bottom of site 1 to site 8 along the length of Sundays Estuary in 2018 and 2019 during sampling in the spring season*

***Table S4:** Statistical results from ANOSIM analysis of sampling sites grouped according to salinity gradient*

	Groups	R-value	p-value	Permutations
2018	Global	0.835	0.001	999
	Polyhaline, Mesohaline	0.651	0.001	999
	Polyhaline, Oligohaline	0.989	0.001	999
	Mesohaline, Oligohaline	0.859	0.001	999
	Global	0.815	0.001	999
2019	Polyhaline, Marine	0.803	0.001	999
	Polyhaline, Mesohaline	0.545	0.001	999
	Polyhaline, Oligohaline	1	0.001	999
	Marine, Mesohaline	0.802	0.001	999
	Marine, Oligohaline	1	0.001	999
	Mesohaline, Oligohaline	0.996	0.001	999

Table S5: *Statistical results on correlation of environmental variables with bacterial distribution patterns using Spearman rank correlation analysis*

	Spearman Rank Correlation						
				BEST			
	Correlation Coefficient (Rho)	p-value	No. of Permutations	Correlation Coefficient (Rho)	p-value	No. of Permutations	Best Correlation variables
2018	0.5	0.001	999	0.965	0.001	999	Dissolved Oxygen, Temperature, TOxN, NH_4, Chl-*a*
2019	0.554	0.001	999	0.828	0.001	999	Salinity, TOxN

Table S6: *Statistical results on significance levels and percentage of bacterial biological variation explained by the individual variables.*

Distance based Linear Modelling (DistLM)				
Year	Variables	p-value	% explained variation	
			Individual	Cumulative
2018	Salinity	0.001	61.58	61.58
	DO	0.024	13.36	74.95
	pH	0.004	2.99	77.94
	Temperature	0.001	1.12	79.06
	ToxN	0.001	0.67	79.74
	SRP	0.001	0.59	80.33
	NH_4	0.002	0.31	80.64
	Chl-*a*	0.013	0.29	80.92
2019	Salinity	0.001	67.64	67.64
	DO	0.001	7.44	75.07
	pH	0.002	3.36	78.44
	Temperature	0.001	1.56	80
	SRP	0.008	1.33	81.33
	ToxN	0.001	0.51	81.84
	NH_4	0.011	0.42	82.26
	Chl-*a*	0.006	0.31	82.58

Table S7: *Blast match results of bacterial OTUs at genus/species level against the GenBank database and Silva_v132 reference database*

OTU	Classification against the Silva_v132 database	Blast analysis against the GenBank nr/nt database			Blast analysis against the GenBank Refseq RNA database		
		Accession Number	Identity Match %	Closest Taxonomic match in NCBI	Accession Number	Identity Match %	Closest Taxonomic match in NCBI
OTU6	*Rheinheimera*	NR_165735.1	98.06%	*Rheinheimera Pleomorphica*	NR_165735.1	98.06%	*Rheinheimera pleomorphica*
OTU7	*Algoriphagus*	EU703226.1	99.27%	*Cyclobacteriaceae*	NR_114262.1	98.29%	*Algoriphagus aquatilis*
OTU10	Candidatus Planktophila	CP016773.1	99.03%	Candidatus planktophila sulfonica	NR_153686.1	90.89%	*Nakamurella silvestris*
OTU12	Uncultured *Cryomorphaceae*	JN639307.1	98.74%	Uncultured bacterium	NR_159281.1	90.24%	*Vicingus serpentipes*
OTU13	*Idiomarina*	MN746254	98.31%	*Idiomarina aestuarii*	NR_116804.1	97.34%	*Idiomarina aestuarii*
OTU14	*Halieaceae*_Unclassified	CP036423.1	99.26%	*Halieceae* bacterium	NR_169349.1	97.05%	*Parahaliea maris*
OTU15	*Rhodobacteraceae*_unclassified	MT112324.1	98.78%	*Phaeobacter gallaeciensis*	NR_152065.1	97.57%	*Sulfitobacter favia*
OTU20	*Rheinheimera*	JQ032270.1	98.54%	Uncultured bacterium	NR_165735.1	98.30%	*Rheinheimera pleomorphica*
OTU22	Uncultured *Cryomorphaceae*	KJ733808.1	97.80%	Uncultured bacterium	NR_136475.1	90.98%	*Phaeocystidibacter marisrubri*
OTU23	NS11-12_marine_group_ge	KT731676.1	98.53%	Uncultured bacterium	NR_044568.1	87.80%	*Solitalea koreensis*
OTU24	NS3a_marine_group	KR077540.1	98.30%	Uncultured bacterium	NR_158088.1	93.70%	*Maribacter elagius*
OTU26	*Burkholderiaceae*_unclassified	AB161308 .1	99.03%	Uncultured bacterium	NR_114131.1	97.57%	*Hydrogenophaga taeniospiralis*
OTU27	Candidatus_Aquiluna	LC314459.1	99.76%	Uncultured *Actinomycetales*	NR_125489.1	98.78%	Candidatus Aquiluna rubra
OTU28	Uncultured *Cryomorphaceae*	JX405652.1	97.80%	Uncultured bacterium	NR_136475.1	91.95%	*Phaeocystidibacter marisrubri*
OTU32	*Exiguobacterium*	FN435982.1	99.52%	Uncultured *Exiguobacterium*	NR_118534.1	99.27%	*Exiguobacterium himgiriensis*
OTU33	NS3a_marine_group	FJ516858.1	99.03%	Uncultured Bacteroidetes	NR_042612.1	95.87%	*Maribacter polysiphoniae*
OTU34	MWH-UniP1_aquatic_group	MK603706.1	98.78%	*Burkholderiale*s bacterium	NR_164971.1	92.94%	*Paraburkholderia strydomiana*
OTU36	XZXXH163	MF040512.1	97.83%	Uncultured bacterium	NR_109611.1	96.62%	*Pontimonas salivibrio*
OTU37	Uncultured_*Cryomorphaceae*	KJ733808.1	98.04%	Uncultured bacterium	NR_136475.1	91.37%	*Phaeocystidibacter marisrubri*

OTU38	*Pseudohongiella*	MK603568.1	98.79%	Gammaproteobacterium	NR_126265.1	96.36%	*Pseudohongiella spirulinae*
OTU39	Unclassified Bacteria	FJ744803.1	95.54%	Uncultured bacterium	NR_044648.2	85.33%	*Absiella tortuosum*
OTU40	NS3a_marine_group	KR077540.1	99.03%	Uncultured bacterium	NR_158088.1	93.70%	*Maribacter pelagius*
OTU41	Hgcl_clade	KM163436.1	99.03%	Uncultured bacterium	NR_043465.1	90.82%	*Demequina aestuarii*
OTU42	Candidatus_Methylopumilus	MT067196.1	98.55%	Uncultured prokaryote	NR_074693.1	94.67%	*Methylotenera versatilis*
OTU43	*Sporichthyaceae_unclassified*	HM129189.1	98.06%	Uncultured bacterium	NR_108784.1	89.45%	*Streptomyces endophyticus*
OTU46	Unclassified Bacteria	FJ744803.1	96.04%	Uncultured bacterium	NR_044648.2	85.33%	*Absiella tortuosum*
OTU48	XZXXH163	MF040512.1	98.07%	Uncultured bacterium	NR_109611.1	96.86%	*Pontimonas salivibrio*
OTU51	RS62_marine_group	DQ234255.2	99.03%	*Comamonadaceae*	NR_114133.1	96.59%	*Hydrogenophaga flava*
OTU52	*Litorimicrobium*	MK603593.1	99.27%	*Rhodobacteraceae*	NR_043928.1	97.32%	*Donghicola eburneus*
OTU54	*Cyanobium_PCC-6307*	KU867943.1	99.03%	*Synechococus* sp.	NR_125481.1	96.37%	*Synechococcus rubescens*
OTU57	*Nostocaceae*_unclassified	KX580772.1	98.31%	*Dolichospermum flos-aquae*	NR_074317.1	92.27%	*Nostoc punctiforme*
OTU58	Candidatus Limnoluna	NR_125497.1	98.79%	Candidatus Limnoluna rubra	NR_125497.1	98.79%	Candidatus Limnoluna rubra
OTU59	MWH-UniP1_aquatic_group	MK603626.1	98.54%	*Burkholderiales* bacterium	NR_164971.1	92.7%	*Paraburkholderia strydomiana*
OTU60	NS11-12_marine_group_ge	FJ612299.1	98.78%	Uncultured bacterium	NR_134125.1	87.29%	*Pedobacter glacialis*
OTU63	NS11-12_marine_group_ge	MT067356.1	98.04%	Uncultured prokaryote	NR_118330.1	88.29%	*Nafulsella turpanensis*
OTU64	MWH-UniP1_aquatic_group	JN634168.1	98.06%	Uncultured Betaproteobacterium	NR_108706.1	92.74%	*Paraburkholderia denitrificans*
OTU65	Clade_III_ge	CP024034.1	99.20%	Candidatus Fonsibacter ubiquis	NR_074224.1	86.10%	Candidatus Pelagibacter ubique
OTU66	*Burkhoderiaceae*_unclassified	EU442993.1	99.03%	Uncultured bacterium	NR_114131.1	98.05%	*Hydrogenophaga taeniospiralis*
OTU67	Clade_Ia	LT840186.1	99.50%	Candidatus Pelagibacter	NR_074224.1	97.80%	Candidatus Pelagibacter ubique
OTU68	NS11-12_marine_group_ge	JQ196109.1	98.53%	Uncultured bacterium	NR_040990.1	89.29%	*Owenweeksia hongkongensis*

APPENDIX 2: Supplementary data for Chapter 4

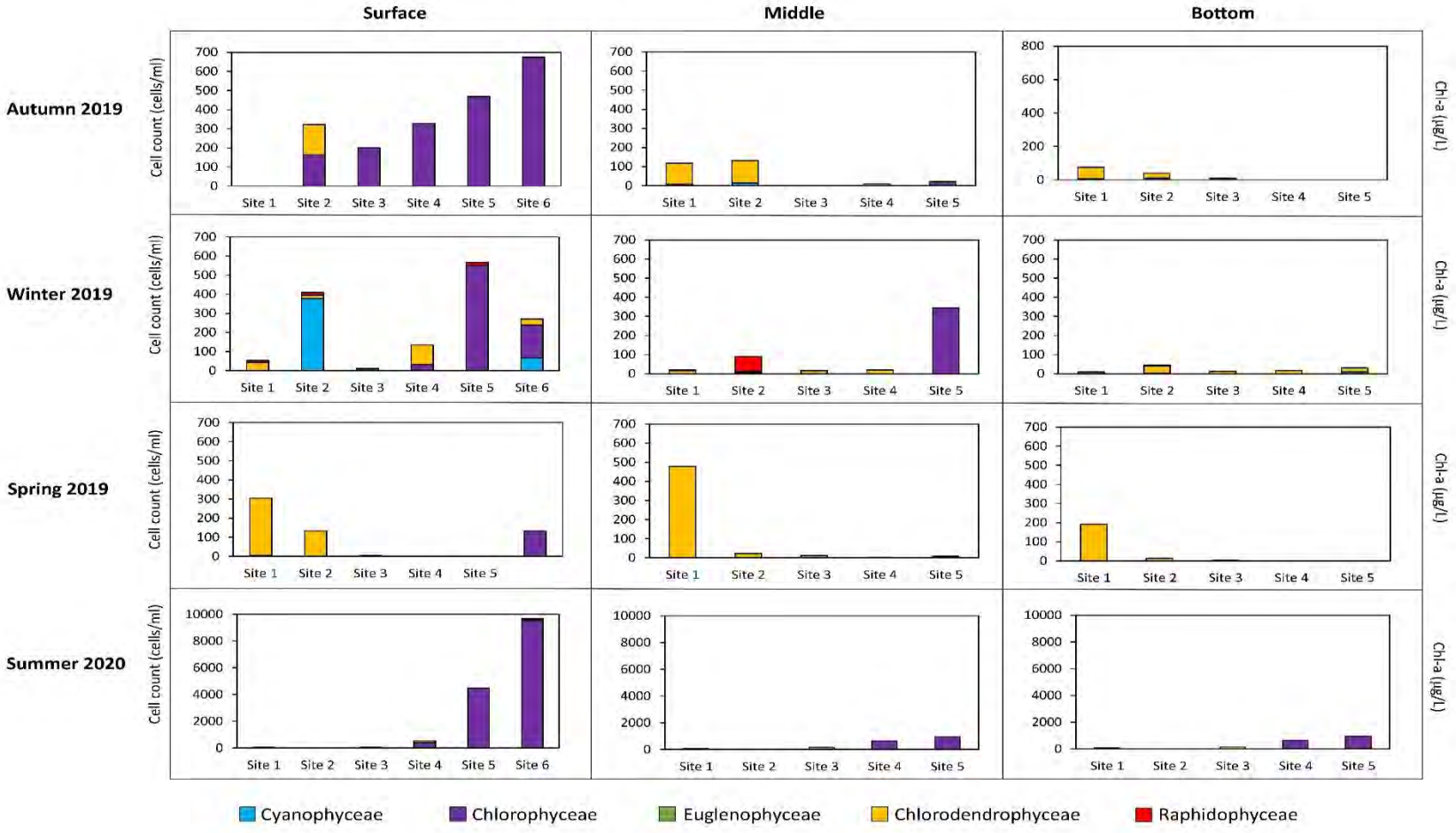

Figure S4: *Non-dominant phytoplankton biomass analysed seasonally through the water column of Swartkops Estuary*

Table S8: *ANOSIM analysis of the bacterioplankton community profiles observed between seasons. Values were calculated using Bray Curtis distance matrix*

	R-value	**p-value**	**Permutations**
Global test	0.376	0.001	999
Autumn, Winter	0.294	0.001	999
Autumn, Spring	0.28	0.001	999
Autumn, Summer	0.396	0.001	999
Winter, Spring	0.286	0.001	999
Winter, Summer	0.571	0.001	999
Spring, Summer	0.438	0.001	999

Table S9: *ANOSIM analysis of the bacterioplankton community profiles observed at each of the sampling sites grouped according to their respective salinity profiles*

Groups	**Autumn**			**Winter**			**Spring**			**Summer**		
	R-value	**p-value**	**Permutations**	**R-value**	**p-value**	**Permutations**	**R-value**	**p-value**	**Permutations**	**R-value**	**p-value**	**Permutations**
Global	0.706	0.001	999	0.55	0.001	999	0.711	0.001	999	0.747	0.001	999
Polyhaline, Mesohaline	0.224	0.04	999	0.29	0.004	999	0.679	0.005	999	0.609	0.001	999
Polyhaline, Oligohaline	1	0.001	999	-	-	-	-	-	-	-	-	-
Polyhaline, Marine	0.689	0.001	999	0.674	0.001	999	0.715	0.001	999	0.717	0.001	999
Mesohaline, Oligohaline	0.675	0.01	999	-	-	-	-	-	-	-	-	-
Mesohaline, Marine	0.877	0.001	999	0.793	0.001	999	0.978	0.011	999	1	0.001	999
Oligohaline, Marine	1	0.001	999	-	-	-	-	-	-	-	-	-

Table S10: *Statistical results on the correlation of environmental variables with bacterial distribution patterns using Spearman rank correlation analysis*

	Spearman Rank Correlation						
	Relate			Best			
Sampling period	Correlation Coefficient (Rho)	p-value	Number of Permutations	Correlation Coefficient (Rho)	p-value	Number of Permutations	Best Correlation variables
Autumn	0.841	0.001	999	0.888	0.001	999	DO, Phosphate, Silicate, Ammonium, TOxN
Winter	0.728	0.001	999	0.922	0.001	999	Salinity, Temperature, Silicate
Spring	0.658	0.001	999	0.853	0.001	999	Salinity, Silicate
Summer	0.718	0.001	999	0.934	0.001	999	Temperature, Phosphate

Table S11*:Statistical results on significance levels and percentage of biological variation explained by the individual variables.*

Distance based Linear Modelling (DistLM)								
	Autumn 2019		Winter 2019		Spring 2019		Summer 2020	
Variables	p-value	% variation explained*	p-value	% variation explained*	p-value	% variation explained*	p-values	% variation explained*
Salinity	0.001	41.02	0.001	48.6	0.001	57.66	0.001	62.04
DO	0.001	23.67	0.022	23.9	0.086	15.88	0.011	18.7
pH	0.002	9.74	0.004	5.18	0.026	6.71	0.001	4.77
Temperature	0.001	2.42	0.001	1.29	0.001	1.64	0.001	1.55
Phosphate	0.001	1.66	0.001	1.13	0.001	1.19	0.001	1.15
Silicate	0.001	0.91	0.001	0.95	0.001	0.88	0.001	0.99
Ammonium	0.001	0.81	0.001	0.82	0.193	0.68	0.46	0.61
ToxN	0.001	0.71	0.001	0.77	0.001	0.55	0.58	0.46
Chl-*a*	0.001	0.66	0.2	0.64	0.026	0.31	0.001	0.3

***Table S12**: Blast match results of bacterial OTUs at genus/species level against the GenBank database and Silva_v132 reference database*

OTUs	Classification against the silva_v132 database	Blast analysis against the GenBank nr/nt database			Blast analysis against the GenBank Refseq RNA database		
		Accession number	Identity match (%)	Closest match in NCBI	Accession number	Identity match (%)	Closest match in NCBI
Otu1	Clade_I_unclassified	KM223510.1	99.27%	Uncultured marine bacterium	NR_074224.1	97.80%	Candidatus Pelagibacteria ubique
Otu2	NS11-12_marine_group	JQ199692.1	99.02%	Uncultured bacterium	NR_044568.1	86.83%	*Solitalea koreensis*
Otu3	*Dinghuibacter*	KM823749.1	99.02%	Uncultured bacterium	NR_164923.1	93.41%	*Flavitaleae flava*
Otu4	*Burkholderiaceae*_unclassified	MT239563.1	98.78%	*Curvibacter* sp.	NR_125544.1	98.54%	*Limnohabitans australis*
Otu5	*Oxyphotobacteria*_unclassified	AY702141.1	98.79%	*Ostreococcus tauri*	XR_002658990.1	77.67%	*Brassica napus*
Otu6	C39	LC132836.1	98.06%	*Rhodocyclaceae* bacterium	NR_028678.1	93.24%	*Azovibrio restrictus*
Otu8	*Rhodobacteraceae*_unclassified	KP262720.1	98.54%	Uncultured bacterium	NR_164623.1	97.08%	*Aliishimia ponticola*
Otu7	C39	LC132836.1	98.55%	*Rhodocyclaceae*_bacterium	NR_028678.1	93.72%	*Azovibrio restrictus*
Otu10	Candidatus_Planktophila	FJ916174.1	98.79%	Uncultured Actinobacterium	NR_153686.1	91.37%	*Nakamurella silvestris*
Otu11	*Flavobacterium*	JQ177676.1	99.02%	Uncultured Flavobacterium sp.	NR_11878.1	98.03%	*Flavobacterium succinicans*
Otu14	*Rhodobacteraceae*_unclassified	KJ870950.1	98.78%	Uncultured *Roseovarius*	NR_152065.1	98.05%	*Sulfitobacter faviae*
Otu15	*Flavobacterium*	KM141914.1	98%	Uncultured bacterium	NR_118476.1	97.54%	*Flavobacterium hydatis*
Otu16	NS5_marine_group	KR077665.1	99.04%	Uncultured bacterium	NR_134750.1	91.33%	*Ichthyenterobacterium magnum*
Otu19	Candidatus_Aquiluna	JX405752.1	98.55%	Uncultured marine bacterium	NR_125497.1	97.34%	Candidatus Limnoluna rubra
Otu20	Clade_Ia	KJ870937.1	99.51%	Uncultured Proteobacterium	NR_074224.1	98.04%	Candidatus Pelagibacter ubique
Otu21	*Cryomorphaceae*_uncultured	DQ656328.1	98.04%	Uncultured Bacteroidetes	NR_136475.1	91.24%	*Phaeocystidibacter marisrubri*
Otu23	*Sporichthyaceae*_ge	MT067415.1	98.79%	Uncultured prokaryote	NR_157616.1	90.41%	*Puzihella rosea*
Otu24	SAR86_clade_ge	KX935461.1	98.79%	Uncultured marine bacterium	NR_114225.1	89.90%	*Pseudomonas mucidolens*
Otu25	Uncultured bacterium	KR077302.1	99.51%	Uncultured bacterium	NR_136475.1	93.19%	*Phaeocystidibacter marisrubri*
Otu26	MWH-UniP1_aquatic_group	KU682047.1	97.81%	Uncultured bacterium	NR_117661.1	92.70%	*Burkholderia pseudomultivorans*
Otu27	*Flavobacterium*	MK158375.1	98.77%	Uncultured Flavobacterium	NR_108893.1	98.04%	*Flavobacterium aquaticum*
Otu28	Uncultured bacterium	JN183361.1	99.02%	Uncultured Bacteroidetes	NR_136475.1	92.44%	*Phaeocystidibacter marisrubri*
Otu29	*Aurantivirga*	KP887673.1	98.02%	Uncultured *Flavobacteriaceae* sp.	NR_025229.1	97.53%	*Tenabaculum skagerrakense*
Otu30	*Sporichthyaceae*_unclassified	HM128912.1	97.82%	Uncultured bacterium	NR_043460.1	89.93%	*Tetrasphaera vanveenii*

Otu31	Gammaproteobacteria_unclassified	MK603700.1	98.06%	Gammaproteobacteria bacterium	NR_043909.1	92.49%	*Modicisalibacter tunisiensis*
Otu32	NS4_marine_group	KP887667.1	98.31%	Uncultured *Flavobacteriaceae*	NR_147772.1	91.35%	*Pseudofulvibacter gastropodicola*
Otu34	NS3a_marine_group	KU173744.1	98.54%	Uncultured Bacteroidetes	NR_158088.1	93.22%	*Maribacter pelagius*
Otu35	SUP05_cluster	KR093353.1	99.26%	Uncultured bacterium	NR_043974.1	90.82%	*Thiohalomonas nitratireducens*
Otu36	HIMB11	KJ870957.1	99.51%	Uncultured *Rhodobacterales*	NR_164623.1	98.05%	*Aliishimia ponticola*
Otu37	NS5_marine_group	KU173746.1	98.31%	Uncultured Bacteroidetes	NR_148332.1	91.13%	*Feifantangia zhejiangensis*
Otu39	*Polynucleobacter*	MT066688.1	99.27%	Uncultured bacterium	NR_151918.1	98.78%	*Polynucleobacter duraquae*
Otu40	*Salinirepens*	MF498459.1	98.53%	Uncultured bacterium	NR_112980.1	95.61%	*Salinirepens amamiensis*
Otu41	*Thioglobaceae*_unclassified	JN233043.1	99.03%	Uncultured Gammaproteobacterium	NR_116014.1	90.29%	*Coxiella cheraxi*
Otu42	Clade_III_ge	KP708781.1	99.03%	Uncultured bacterium	NR_116131.1	98.05%	*Acidovorax delafieldii*
Otu43	*Chromatiaceae*_unclassified	FJ745136.1	98.31%	Uncultured Gammaproteobacteria	NR_148757.1	92.03%	*Thiolapillus brandeum*
Otu44	OM43_clade	KJ870956.1	99.03%	Uncultured *Nitrosomonadales*	NR_074693.1	96.37%	*Methylotenera versatilis*
Otu45	*Pseudarcicella*	FN668111.2	99%	Uncultured *Flectobacillus* sp.	NR_165029.1	98.76%	*Aquirufa antheringensis*
Otu47	Candidatus_Actinomarina	JQ196062.1	98.80%	Uncultured bacterium	NR_028778.1	82.62%	*Acidipropionibacterium microaerophilum*
Otu48	*Marinobacterium*	KF146347.1	99.51%	*Marinobacterium* sp.	NR_125520.1	98.30%	*Marinobacterium marisflavi*
Otu49	*Arcobacter*	AB205716.1	98.79%	Uncultured bacterium	NR_042218.1	99%	*Arcobacter cibarius*
Otu50	Clade_III_ge	MK603699.1	98.53%	*Pelagibacterales* bacterium	NR_074224.1	86.83%	Candidatus Pelagibacter ubique
Otu51	Uncultured bacterium	KT731841.1	98.02%	Uncultured Bacteroidetes	NR_159212.1	93.67%	*Frondibacter mangrovi*
Otu52	*Burkholderiaceae*_unclassified	MH085276.1	98.54%	Uncultured bacterium	NR_114131.1	97.32%	*Hydrogenophaga taeniospiralis*
Otu56	NS3a_marine_group	KR077661.1	98.79%	Uncultured bacterium	NR_135863.1	93.46%	*Algibacter psychrophilus*
Otu58	*Burkholderiaceae*_unclassified	MT239563.1	98.30%	*Curvibacter* sp.	NR_125544.1	98.05%	*Limnohabitans australis*
Otu59	NS3a_marine_group	FJ516858.1	98.30%	Uncultured Bacteroidetes	NR_042612.1	95.63%	*Maribacter polysiphoniae*
Otu61	Chloroplast_ge	MT111931.1	98.79	*Ostreococcus* sp. *'lucimarinus'*	XR_002658990.1	77.49%	*Brassica napus*
Otu63	*Litorimicrobium*	KR077725.1	98.05%	Uncultured bacterium	NR_125550.1	96.35%	*Planktomarina temperata*
Otu64	*Burkholderiaceae*_unclassified	KM163175.1	98.78%	Uncultured bacterium	NR_125491.1	98.54%	*Limnohabitans curvus*
Otu65	*Burkholderiaceae*_unclassified	HF968558.1	99.51%	Uncultured *Limnohabitans*	NR_125541.1	99.27%	*Limnohabitans planktonicus*
Otu66	MWH-UniP1_aquatic_group	MK603706.1	97.32%	*Burkholderiales* bacterium	NR_164971.1	91.24%	*Paraburkholderia strydomiana*

Otu67	RS62_marine_group	MT950111.1	99.51%	*Hydrogenophaga* sp.	NR_114133.1	97.32%	*Hydrogenophaga* sp.
Otu68	*Amylibacter*	KU173621.1	98.30%	Uncultured Alphaproteobacterium	NR_146351.1	97.38%	*Amylibacter ulvae*
					NR_13396.1		*Amylibacter marinus*
Otu69	Uncultured bacterium	MW559863.1	99.49%	Uncultured bacterium	NR_044346.1	93.64%	*Joostella marina*
Otu72	Clade_Ia	JQ199329.1	98.53%	Uncultured bacterium	NR_074224.1	97.80%	Candidatus Pelagibacter ubique
Otu73	*Rhodobacterraceae*_unclassified	NR_074150.1	99.51%	*Ruegeria pomeroyi*	NR_074150.1	99.51%	*Ruegeria pomeroyi*
Otu76	NS3a_marine_group	KU173779.1	99.03%	Uncultured Bacteroidetes/Chlorobi	NR_136467.1	93.96%	*Maribacter spongiicola*
Otu78	*Fluviicola*	JN591937.1	98.04%	Uncultured *Cryomorphaceae*	NR_116229.1	92.70%	*Lishizhenia tianjinesis*
Otu80	NS5_marine_group	FJ744872.1	97.83%	Uncultured Flavobacterium	NR_149793.1	90.41%	*Kordia ulvae*
Otu81	Uncultured bacterium	MF498311.1	99.02%	Uncultured bacterium	NR_136475.1	92.44%	*Phaeocystidibacter marisrubri*
Otu82	*Amylibacter*	KU173621.1	98.05%	Uncultured Alphaproteobacterium	NR_146351.1	97.08%	*Amylibacter ulvae*
					NR_133962.1		*Amylibacter marinus*
Otu83	*Arthrospira_PCC-7345*	MT426015.1	98.31%	*Arthrospira platensis BEA1257B*	NR_125711.1	97.58%	*Arthrospira platensis*
Otu84	*Idiomarina*	MN746254.1	98.31%	*Idiomarina aestuarii*	NR_116804.1	97.34%	*Idiomarina aestuarii*
Otu85	*Methylophilaceae*_unclassified	KP687229.1	99.76%	Uncultured bacterium	NR_104760.1	97%	*Mathylobacillus_glycogenes*
Otu88	Clade_Ia	KR077685.1	99.27%	Uncultured bacterium	NR_074224.1	98.53%	Candidatus Pelagibacter ubique
Otu89	SAR86_clade_ge	KR077656.1	99.27%	Uncultured bacterium	NR_029050.1	90.14%	*Pseudomonas palleroniana*
Otu90	*Oxyphotobacteria*_unclassified	KR077740.1	99.28%	Uncultured bacterium	XM_027503725	77.49%	*Abrus precatorius*
Otu91	Bacteroidetes_uncultured	JN232997.1	99.27%	Uncultured Flavobacterium sp.	NR_132329.1	92.70%	*Phaeocystidibacter luteus*
Otu92	*Burkholderiaceae*_unclassified	FQ660437.1	98.77%	Uncultured soil bacterium	NR_029024.1	98.28%	*Hydrogenophaga defluvii*
Otu93	*Litoricola*	MK603737.1	98.06%	Gammaproteobacteria bacterium	NR_104279.1	93.24%	*Pseudomonas cremoricolorata*
Otu94	*Amylibacter*	JQ194950.1	98.78%	Uncultured bacterium	NR_146351.1	97.81%	*Amylibacter ulvae*
Otu95	*Litorimicrobium*	MK603593.1	98.54%	*Rhodobacteraceae* bacterium	NR_043928.1	96.51%	*Donghicola erburneus*
Otu96	*Cyanobium_PCC-6307*	MT488300.1	99.03%	*Cyanobium* sp. CHAB 6568	NR_102447.1	99.03%	*Cyanobium gracile PCC 6307*
Otu98	Uncultured bacterium	LR639372.1	98.54%	Uncultured bacterium	NR_114615.1	93.93%	*Nordella oligomobilis*
Otu99	Clade_Ia	KJ870937.1	98.78%	Uncultured Proteobacterium	NR_074224.1	97.31%	Candidatus Pelagibacter ubique
Otu100	Clade_Ia	CP031125.1	99.02%	Candidatus Pelagibacter	NR_074224.1	98.53%	Candidatus Pelagibacter ubique
Otu101	*Flavobacteriaceae*_unclassified	HE979562.1	98.77%	Uncultured bacterium	NR_149768.1	97.79%	*Tenacibaculum sediminilitoris*

Otu105	AEGEAN-169_marine group	KU382423.1	100%	*Pelagibacterales* bacterium	NR_153725.1	85.44%	*Iodidimonas muriae*
Otu107	*Flavobacterium*	KU529208.1	98.77%	*Flavobacterium* sp.	NR_158093.1	97.54%	*Flavobacterium ardleyense*
Otu108	C39	EU234274.2	98.55%	Uncultured bacterium	NR_028678.1	94.44%	*Azovibrio restrictus*
Otu109	*Fluviicola*	AJ965997.1	98.04%	Uncultured bacterium	NR_112980.1	93.17%	*Salinirepens amamiensis*
Otu111	*Formosa*	MF040084.1	99.27%	Uncultured *Formosa* sp.	NR_125695.1	97.80%	*Formosa arctica*
Otu112	NS11-12_marine_group	DQ080954.1	98.76%	Uncultured Bacteroidetes	NR_133875.1	87.14%	*Taibaiella chishuiensis*
Otu115	*Rhodobacteraceae*_unclassified	AF194383.1	98.78%	Uncultured Alphaproteobacteria	NR_108333.1	98.54%	*Lentibacter algarum*
Otu116	NS11-12_marine_group_ge	AM279196.1	98.04%	Uncultured Sphingobacteria	NR_043917.1	86.10%	*Marinoscillum pacificum*
Otu117	*Polynucleobacter*	HQ111147.1	98.54%	Uncultured *Polynucleobacter*	NR_151920.1	98.30%	*Polynucleobacter sinensis*
Otu118	Bacteria_unclassified	LM652163.1	98.55%	Uncultured protist	XR_002658990.1	77.73%	*Brassica napus*
Otu121	*Polaribacter_4*	JX304645.1	98.53%	*Polaribacter* sp.	NR_043456.1	98.28%	*Polaribacter dokdonensis DSW-5*
Otu123	NS3a_marine_group	KU173779.1	98.79%	Uncultured Bacteroidetes/Chlorobi	NR_025749.1	93.72%	*Maribacter orientalis*
Otu126	SAR116_clade_*unclassified*	KM223948.1	99%	Uncultured marine bacterium	NR_044165.1	90.75%	*Thalassobaculum litoreum*
Otu127	HIMB11	KR077550.1	98.54%	Uncultured bacterium	NR_164623.1	97.32%	*Aliishimia ponticola*
Otu129	*Flavobacterium*	KU529208.1	98.28%	*Flavobacterium* sp.	NR_158093.1	97.54%	*Flavobacterium ardleyense*
Otu130	*Flavobacteriaceae*_unclassified	JQ200017.1	98.29%	Uncultured bacterium	NR_109551.1	97.07%	*Olleya namhaensis*
Otu131	SAR11_clade_unclassified	EU801709.1	99.27%	Uncultured bacterium	NR_074224.1	94.15%	Candidatus Pelagibacter ubique
Otu132	*Planktomarina*	HE572717.1	100%	Uncultured bacterium	NR_125550.1	99.75%	*Planktomarina temperata*
Otu133	*Microbacteriaceae*_unclassified	CP026923.1	98.55%	*Pontimonas salivibrio*	NR_109611.1	98.31%	*Pontimonas salivibrio*
Otu134	NS9_marine_group_ge	EF016485.1	98.52%	Uncultured Bacteroidetes bacterium	NR_114284.1	85.85%	*Wandonia_haliotisNBRC 105640*
Otu136	Clade_Ia	AF194383.1	99.26%	Uncultured Alphaproteobacteria	NR_074224.1	98.04%	Candidatus Pelagibacter ubique
Otu137	*Aureimarina*	EF108215.1	98.78%	*Aureimarina marisflavi*	NR_109381.1	93.64%	*Gaetbulibacter lutimaris*
Otu138	NS4_marine_group	KX936581.1	99.27%	Uncultured bacterium	NR_147772.1	92.18%	*Pseudofulvibacter gastropodicola*
Otu139	*Flavobacteriales*_unclassified	FJ524893.1	97.93%	Uncultured bacterium	NR_136481.1	90%	*Elizabethkingia anophelis*
Otu140	*Aurantivirga*	NR_157991.1	98.77%	*Polaribacter pacificus*	NR_157991.1	98.77%	*Polaribacter pacificus*
Otu141	Uncultured bacterium	KX936675.1	99.27%	Uncultured marine bacterium	NR_132329.1	92.94%	*Phaeocystidibacter luteus*
Otu142	*Pseudohongiella*	MK603568.1	98.54%	Gammaproteobacteria bacterium	NR_126265.1	96.36%	*Pseudohongiella spirulinae*
Otu143	*Cyanobium_PCC-6307*	DQ228155.1	99.52%	Uncultured *Synechococcus* sp.	NR_102447.1	97.82%	*Cyanobium gracile PCC 6307*

Otu144	*Cyanobium_PCC-6307*	AB795492.1	98.55%	Uncultured *Synechococcus* sp.	NR_102447.1	97.34%	*Cyanobium gracile PCC 6307*
Otu145	*Pirellulaceae*_unclassified	LR637527.1	96.37%	Uncultured bacterium	NR_136448.1	93.70%	*Rhodopirellula caenicola*
Otu146	Luteolibacter	EU182121.2	98.78%	Uncultured bacterium	NR_109500.1	93.70%	*Luteolibacter luojiensis*
Otu147	*Cyanobium_PCC-6307*	JX672087.1	99.52%	Uncultured bacterium	NR_125481.1	97.82%	*Synechococcus rubescens*
Otu148	Mitochondria_ge	FJ353764.1	96.64%	Uncultured organism	NR_025606.1	73.25%	*Helcococcus sueciensis*
Otu149	*Flavobacteriaceae*_unclassified	KX934667.1	99.51%	Uncultured marine bacterium	NR_133820.1	99.02%	*Polaribacter atrinae*
Otu150	*Halioglobus*	EU592391.1/ EU592383.1	99.51%	Uncultured bacterium	NR_169349.1	96.60%	*Parahaliea maria*
Otu151	*Cyanobium_PCC-6307*	KR077621.1	99.03%	Uncultured bacterium	NR_125481.1	96.13%	*Synechococcus rubescens*
Otu152	Proteobacteria_unclassified	FJ208405.1	99.27%	Uncultured bacterium	NR_159917.1	74.17%	*Litorimonas haliclonae*
Otu153	*Saprospiraceae*_uncultured	KF798751.1	99.02%	Uncultured bacterium	NR_028695.1	89.02%	*Flavilitoribacter nigricans*
Otu154	Bacteroidia_unclassified	KJ914567.1	98.53%	Uncultured bacterium	NR_074100.1	92.68%	*Owenweeksia hongkongensis*
Otu155	*Cyanobium_PCC-6307*	FJ208405.1	99.27%	Uncultured bacterium	NR_102447.1	97.34%	*Cyanobium gracile PCC 6307*

APPENDIX 3: Supplementary data for Chapter 5

Table S13: Physico-chemical variables recorded in canals, rivers and creek at the time of sampling in the autumn season (2019)

Canals	**Temperature (˚C)**	**pH**	**% DO**
Tippers creek	18	7.7	69.7
Chatty river	18.6	8	63.7
Markman canal	20.6	8	99.6
Motherwell canal	20.7	7.9	18.2
Perseverance bridge	18.8	7.4	17.3
Nivens bridge	19.4	7.5	38.8
Bulmers drift	20.7	7.1	94.8
Springfontein	22.8	7.4	122.6

Table S14: *Statistical results from ANOSIM analysis of bacterial OTUs generated from the water column of Swartkops Estuary and inflow from canals, rivers, and creek against selected variables. Values were calculated using Bray Curtis distance matrix.*

			R-Statistics	p-value	Permutations
A	Phosphorus Concentration	Global	0.712	0.001	999
		Eutrophic, Mesotrophic	0.681	0.001	30260340
		Eutrophic, Hypertrophic	0.738	0.001	472733756
		Eutrophic, Oligotrophic	0.955	0.002	435
		Mesotrophic, Hypertrophic	0.516	0.004	43758
		Mesotrophic, Oligotrophic	0.272	0.111	45
		Hypertrophic, Oligotrophic	0.422	0.061	66
B	Location	Global	0.693	0.001	999
		Upper Estuary, Lower Canal	0.452	0.014	495
		Upper Estuary, Upper Canal	0.474	0.001	6435
		Upper Estuary, Middle Canal	0.778	0.002	495
		Middle Estuary, Lower Canal	0.795	0.002	1820
		Middle Estuary, Upper Canal	0.94	0.001	125970
		Middle Estuary, Middle Canal	1	0.003	1820
		Lower Estuary, Lower Canal	0.435	0.017	1820
		Lower Estuary, Upper Canal	0.988	0.001	125970
		Lower Estuary, Middle Canal	1	0.002	1820
		Lower Canal, Upper Canal	1	0.002	495
		Lower Canal, Middle Canal	1	0.029	35
		Upper Canal, Middle Canal	0.64	0.004	495
C	Nitrogen Concentration	Global	0.578	0.001	999
		Mesotrophic, Oligotrophic	0.491	0.001	Very large
		Mesotrophic, Eutrophic	0.748	0.002	561
		Mesotrophic, Hypertrophic	0.884	0.002	561
		Oligotrophic, Eutrophic	0.261	0.099	91
		Oligotrophic, Hypertrophic	0.301	0.088	91
		Eutrophic, Hypertrophic	1	0.333	3

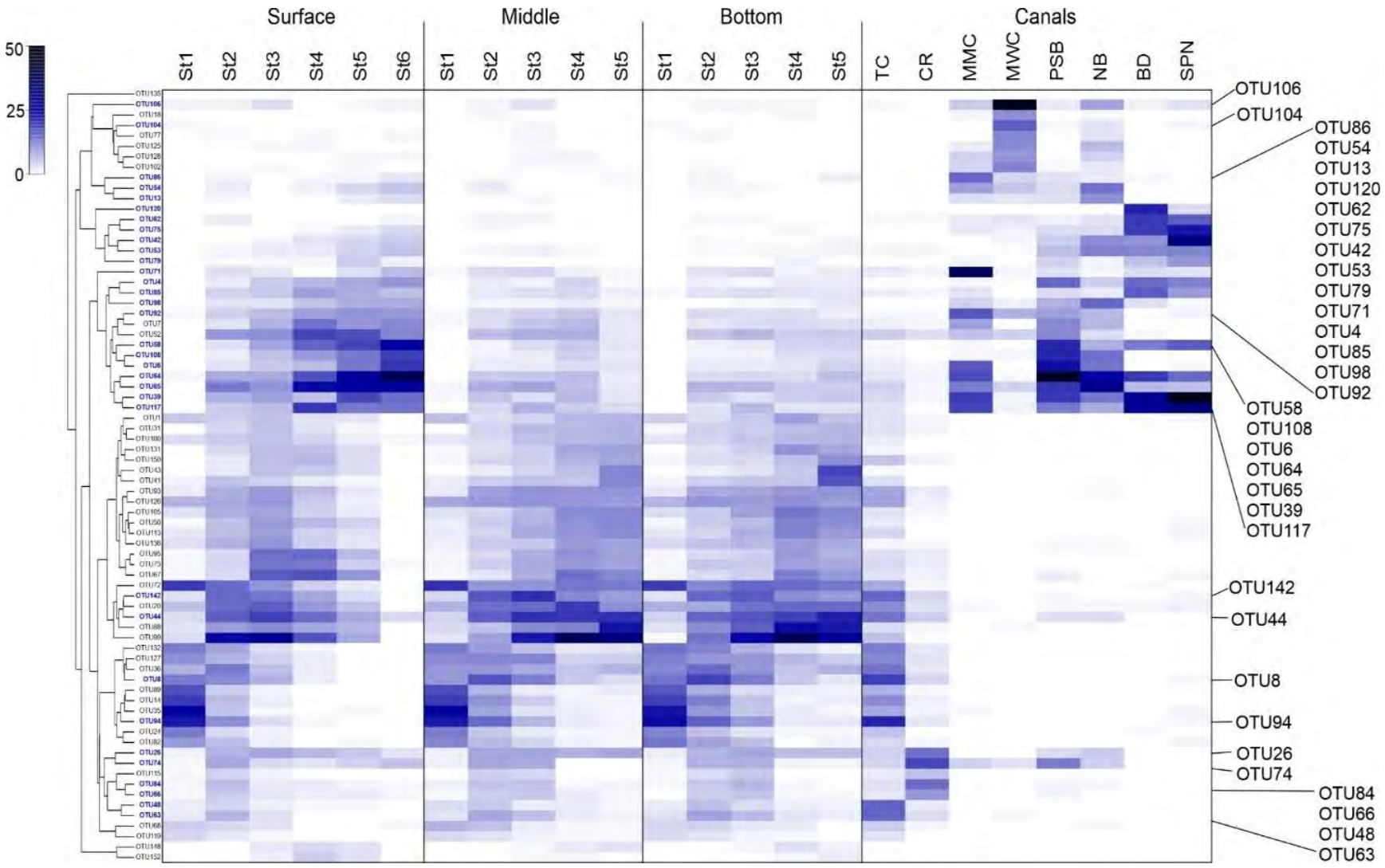

Figure S5A: Heatmap of the top 10 most dominant bacterial OTUs in the Proteobacteria phylum, showing inflow from canals, rivers, and creek into Swartkops Estuary at the time of sampling in the autumn season (2019). OTUs were clustered at distance of 0.03 and data was transformed to Square Root and standardised. OTUs of interest are highlighted in blue color.

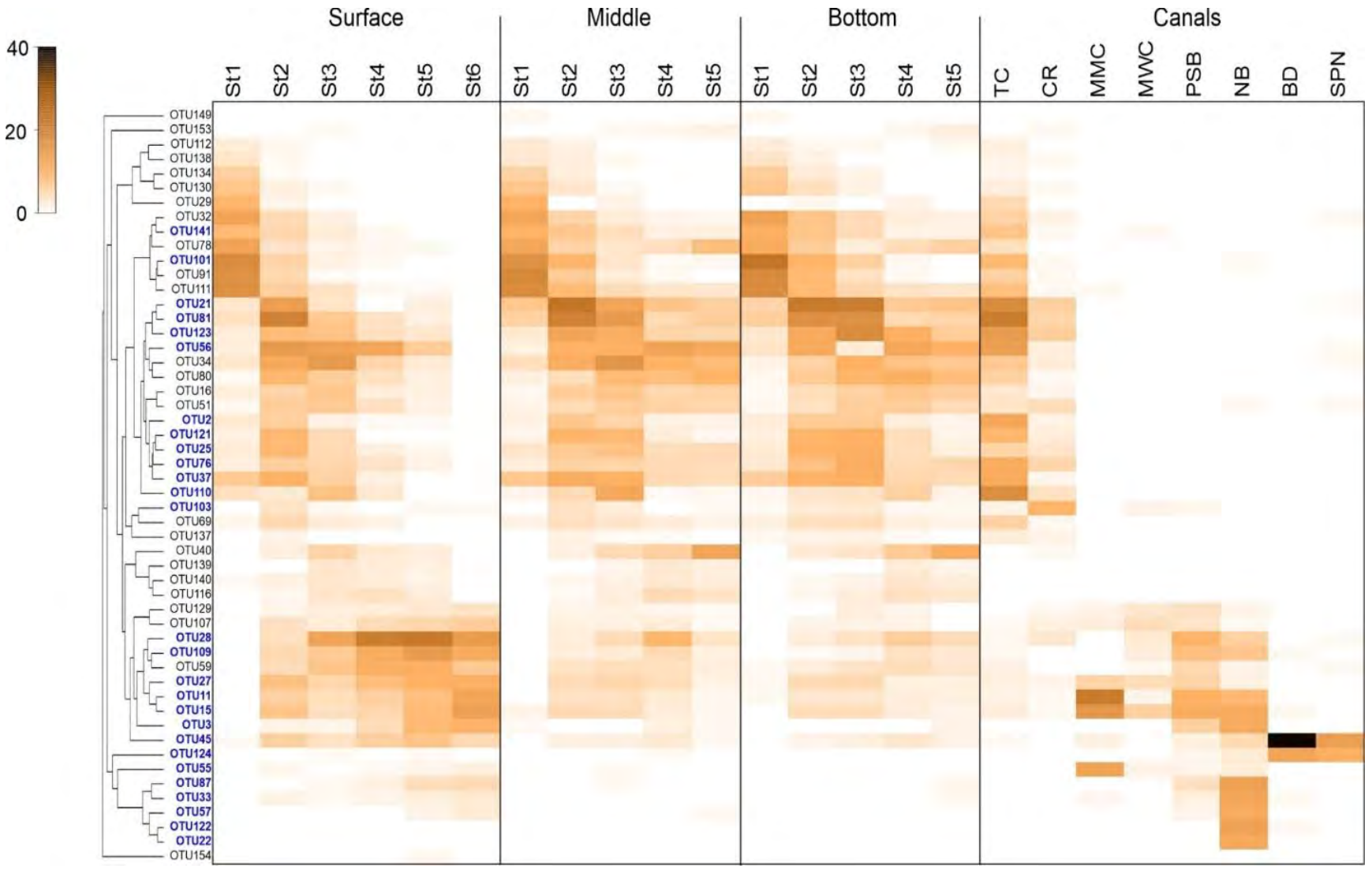

Figure S5B: Heatmap of the top 10 most dominant bacterial OTUs in the Bacteroidetes phylum, showing inflow from canals, rivers, and creek into Swartkops Estuary at the time of sampling in the autumn season (2019). OTUs were clustered at distance of 0.03 and data was transformed to Square Root and standardised. OTUs of interest are highlighted in blue color.

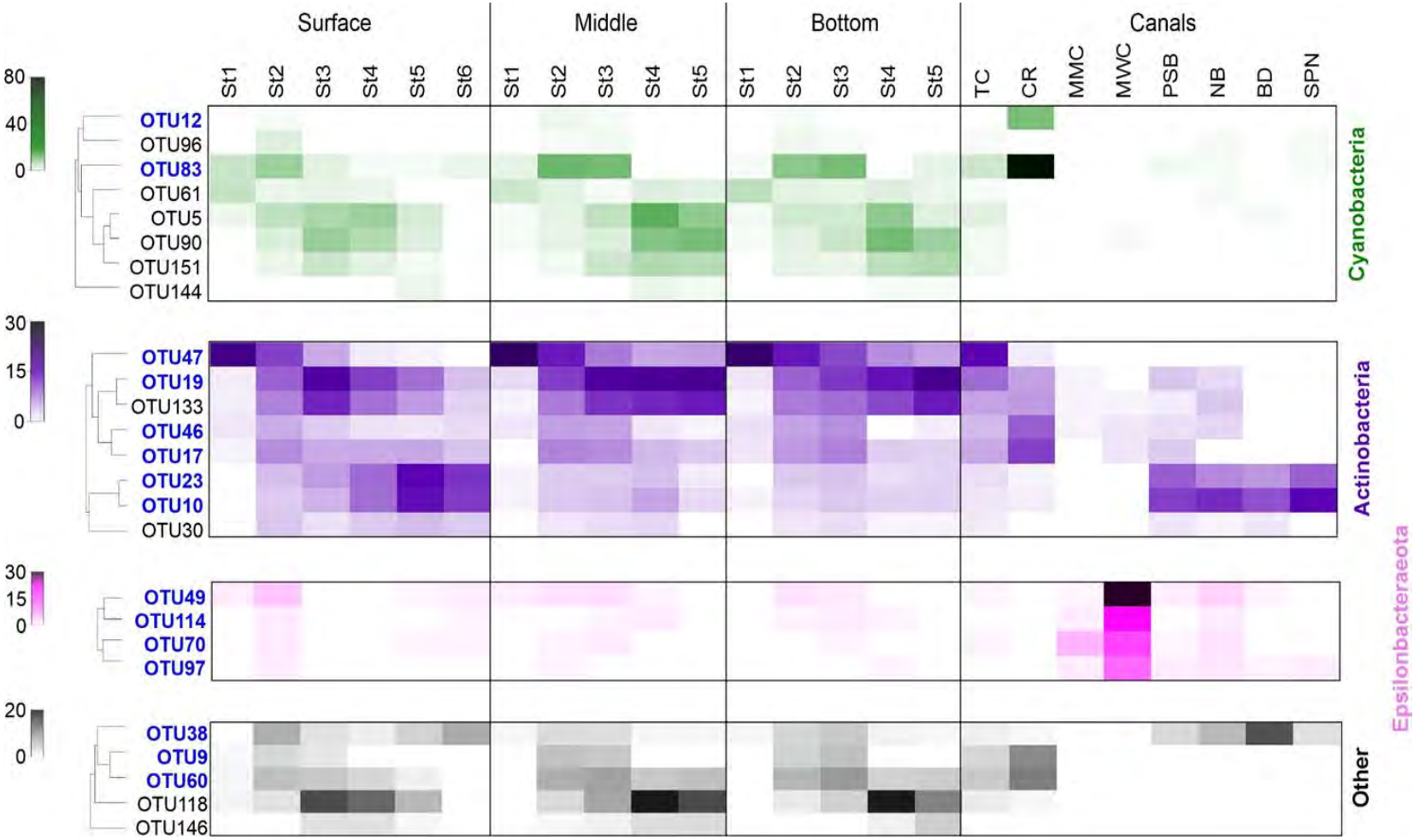

Figure S5C: Heatmap of the top 10 most dominant bacterial OTUs in different phyla showing inflow from canals, rivers, and creek into Swartkops Estuary at the time of sampling in the autumn season (2019). OTUs were clustered at distance of 0.03 and data was transformed to Square Root and standardised. OTUs of interest are highlighted in blue color.

Table S15: *Blast match results of bacterial OTUs at genus/species level against the GenBank database and Silva_v132 reference database*

OTUs	Sequence Match against the Silva v132 reference database	Blast analysis against the GenBank nr/nt database			Blast analysis against the GenBank Refseq RNA database		
		Accession number	Identity match %	Closest match in NCBI	Accession number	Identity match %	Closest match in NCBI
Otu2	NS11-12_marine_group	JQ199692.1	99.02%	Uncultured bacterium	NR_044568.1	86.83%	*Solitalea koreensis*
Otu3	*Flavobacterium*	KM823749.1	98.04%	Uncultured bacterium	NR_164923.1	93.41%	*Flavitalea flava*
Otu4	*Burkholderiaceae_unclassified*	MT239563.1	98.78%	*Curvibacter* sp.	NR_125544.1	98.54%	*Limnohabitans australis*
Otu6	C39	LC132836.1	98.06%	*Rhodocyclaceae bacterium*	NR_028678.1	93.24%	*Azovibrio restrictus*
Otu8	*Rhodobacteraceae_unclassified*	KP262720.1	98.54%	Uncultured bacterium	NR_164623.1	97.08%	*Aliishimia ponticola*
Otu9	*Izimaplasmataceae*	JN117222.1	97%	Uncultured bacterium	NR_109459.1	87.41%	*Alkalihalobacillus berkeleyi*
Otu10	Candidatus_Planktophila	FJ916174.1	98.79%	Uncultured *Actinobacterium*	NR_153686.1	91.37%	*Nakamurella silvestris*
Otu11	*Flavobacterium*	JQ177676.1	99.02%	Uncultured *Flavobacterium*	NR_118478.1	98.03%	*Flavobacterium siccinicans*
Otu12	*Arthrospira_PCC-7345*	MT426015.1	98.06%	*Arthrospira platensis*	NR_125711.1	97.34%	*Arthrospira platensis*
Otu13	*Burkholderiaceae_unclassified*	JN371497.1	99.03%	Uncultured *Betaproteobacterium*	NR_165749.1	98.30%	*Tepidicella baoligensis*
Otu14	*Rhodobacteraceae_unclassified*	KJ870950.1	98.78%	Uncultured *Roseovarius*	NR_152065.1	98.05	*Sulfitobacter faviae*
Otu15	*Flavobacterium*	KM141914.1	98%	Uncultured bacterium	NR_118476.1	97.54%	*Flavobacterium hydatis*
Otu17	Candidatus_aquiluna	KJ733804.1	99.03%	Uncultured bacterium	NR_125489.1	98.07%	Candidatus Aquiluna rubra
Otu18	*Shewanella*	CU467450.1/ DQ088226.1	98.79%	Uncultured bacterium	NR_113582.1/NR_044863.1/NR_119141.1 NR_104770.1 NR_116732.1	98.54%	*Shewanella putrefaciens* *Shewanella profunda* *Shewanella xiamenensis*
Otu19	Candidatus_Aquiluna	JX405752.1	98.55%	Uncultured marine bacterium	NR_125497.1	97.34%	Candidatus Limnoluna rubra
Otu20	Clade _Ia	KJ870937.1	99.51%	Uncultured Proteobacterium	NR_074224.1	98.04%	Candidatus Pelagibacteria ubique
Otu21	*Cryomorphaceae_uncultured*	DQ656328.1	98.04%	Uncultured *Bacteroidetes*	NR_136475.1	91.24%	*Phaeocystidibacter marisrubri*
Otu22	*Flavobacterium*	MN334777.1	98.77%	*Flavobacterium* sp.	NR_157714.1	98.03%	*Flavobacterium fluminis*
Otu23	*Sporichthyaceae*_ge	MT067415.1	98.79%	Uncultured prokaryote	NR_157616.1	90.41%	*Puzihella rosea*
Otu25	*Uncultured Cryomorphaceae*	KR077302.1	99.51%	Uncultured bacterium	NR_136475.1	93.19%	*Phaeocystidibacter marisrubri*
Otu26	MWH-UniP1_aquatic_group	KU682047.1	97.81%	Uncultured bacterium	NR_117661.1	92.70%	*Burkholderia pseudomultivorans*

Otu27	*Flavobacterium*	MK158375.1	98.77%	Uncultured *Flavobacterium*	NR_108893.1	98.04%	*Flavobacterium aquaticum*
Otu28	*Cryomorphaceae_uncultured*	JN183361.1	99.02%	Uncultured *Bacteroidetes*	NR_136475.1	92.44%	*Phaeocystidibacter marisrubri*
Otu33	*Flavobacterium*	FR746079.1	98.28%	*Flavobacterium* sp.	NR_158092.1	97.54%	*Flavobacterium quisquiliarum*
Otu36	HIMB11	KJ870957.1	99.51%	uncultured Rhodobacterales	NR_164623.1	98.05%	*Aliishimia ponticola*
Otu37	NS5_marine_group	KU173746.1	98.31%	Uncultured *Bacteroidetes*	NR_148332.1	91.13%	*Feifantangia zhejiangensis*
Otu38	Uncultured_ge	MT067369.1	99.52%	Uncultured prokaryote	NR_157674.1	87.50%	*Ereboglobus luteus*
Otu39	*Polynucleobacter*	MT066688.1	99.27%	Uncultured bacterium	NR_151918.1	98.78%	*Polynucleobacter duraquae*
Otu42	Clade_III_ge	KP708781.1	99.03%	Uncultured bacterium	NR_116131.1	98.05%	*Acidovorax delafieldii*
Otu44	OM43_clade	KJ870956.1	99.03%	Uncultured *Nitrosomonadales*	NR_074693.1	96.37%	*Methylotenera versatilis*
Otu45	*Pseudarcicella*	FN668111.2	99.01%	Uncultured *Flectobacillus*	NR_165029.1	98.76%	*Aquirufa antheringensis*
Otu46	PeM15_ge	LR641432.1	99.28%	Uncultured *bacterium*	NR_159886.1	95.41%	*Longivirga aurantiaca*
Otu47	Candidatus_Actinomarina	JQ196062.1	98.80%	*Uncultured bacterium*	NR_028778.1	82.62%	*Acidipropionibacterium microaerophilum*
Otu48	*Marinobacterium*	KF146347.1	99.51%	*Marinobacterium* sp.	NR_125520.1	98.30%	*Marinobacterium marisflavi*
Otu49	*Arcobacter*	AB205716.1	98.79%	Uncultured *bacterium*	NR_042218.1	99%	*Arcobacter cibarius*
Otu52	*Burkholderiaceae_unclassified*	MH085276.1	98.54%	Uncultured bacterium	NR_114131.1	97.32%	*Hydrogenophaga taeniospiralis*
Otu53	*Burkholderiaceae_unclassified*	LC217413.1	98.54%	*Betaproteobacterium*	NR_108708.1	98.30%	*Caenimonas terrae*
Otu54	*Pseudohongilla*	HG917750.1	98.54%	Uncultured bacterium	NR_153732.1	96.60%	*Pseudohongiella nitratireducens*
Otu55	*Flavobacterium*	MK824758.1	98.28%	Bacterium	NR_159129.1	97.79%	*Flavobacterium limi*
Otu56	NS3a_marine_group	KR077661.1	98.79%	Uncultured bacterium	NR_135863.1	93.46%	*Algibacter psychrophilus*
Otu57	*Runella*	JN371411.1	98.78%	Uncultured Bacteroidetes	NR_043771.1	96.82%	*Runella limosa*
Otu58	*Burkholderiaceae_unclassified*	MT239563.1	98.30%	*Curvibacter* sp.	NR_125544.1	98.05%	*Limnohabitans australis*
Otu60	Bacteria_unclassified	LT800257.1	98.27%	Uncultured bacterium	NR_044362.1	87.01%	*Haloplasma contractile SSD-17B*
Otu62	*Burkhodericeae unclassified*	AY360707.1	99.27%	Uncultured *Comamonadaceae*	NR_133836.1	99.03%	*Ramlibacter ginsenosidimutans*
Otu63	*Litorimicrobium*	KR077725.1	98.05%	Uncultured bacterium	NR_125550.1	96.35%	*Planktomarina temperata*
Otu64	*Burkholderiaceae_unclassified*	KM163175.1	98.78%	Uncultured bacterium	NR_125491.1	98.54%	*Limnohabitans curvus*
Otu65	*Burkholderiaceae_unclassified*	HF968558.1	99.51%	Uncultured *Limnohabitans*	NR_125541.1	99.27%	*Limnohabitans planktonicus*

Otu66	MWH-UniP1_aquatic_group	MK603706.1	97.32%	*Burkholderiales* bacterium	NR_164971.1	91.24%	*Paraburkholderia strydomiana*
Otu70	*Arcobacter*	JX912354.1	99.01%	Uncultured *Arcobacter*	NR_117570.1	98.52%	*Arcobacter cloacae*
Otu71	C39	LC132788.1	99.03%	*Rhodocyclaceae bacterium*	NR_028678.1	96.61%	*Azovibrio restrictus*
Otu72	Clade_la	JQ199329.1	98.53%	Uncultured bacterium	NR_074224.1	97.80%	Candidatus Pelagibacteria ubique
Otu74	*Burkholderiaceae_unclassified*	KX163849.1	99.03%	Uncultured bacterium	NR_158145.1	98.78%	*Hydrogenophaga soli*
Otu75	*Burkholderiaceae_unclassified*	CP054840.1	99.03%	*Acidovorax* sp.	NR_113696.1	98.78%	*Curvibacter delicatus*
Otu76	NS3a_marine_group	KU173779.1	99.03%	Uncultured *Bacteroidetes/Chlorobi group bacterium*	NR_136467.1	93.96%	*Maribacter spongiicola*
Otu79	*Polynucleobacter*	CP028942	98.00%	*Polynucleobacter* sp.	NR_151920.1	98.00%	*Polynucleobacter sinensis*
Otu77	*Macromonas*	KM668168.1	98.78%	Uncultured bacterium	NR_040903.1	98.06%	*Macromonas bipunctata*
Otu81	Uncultured *Cryomorphaceae*	MF498311.1	99.02%	Uncultured bacterium	NR_136475.1	92.44%	*Phaeocystidibacter marisrubri*
Otu83	*Arthrospira_PCC-7345*	MT426015.1	98.31%	*Arthrospira platensis*	NR_125711.1	97.58%	*Arthrospira platensis*
Otu84	*Idiomarina*	MN746254.1	98.31%	*Idiomarina aestuarii*	NR_116804.1	97.34%	*Idiomarina aestuarii*
Otu85	*Methylophilaceae_unclassified*	KP687229.1	99.76%	Uncultured bacterium	NR_104760.1	97.00%	*Methylobacillus glycogenes*
Otu86	*Thiobacillus*	GQ860185.1	99.27%	Uncultured bacterium	NR_115758.1	98.31%	*Thiobacillus sajanensis*
Otu87	*Spirosomaceae*_ge	MH463956.2	98.78%	*Fluviimonas* sp.	NR_117642.1	96.10%	*Fluviimonas pallidilutea*
Otu92	*Burkholderiaceae_unclassified*	FQ660437.1	98.77%	Uncultured soil bacterium	NR_029024.1	98.28%	*Hydrogenophaga defluvii*
Otu93	*Litoricola*	MK603737.1	98.06%	Gammaproteobacterium bacteria	NR_104279.1	93.24%	*Pseudomonas cremoricolorata*
Otu94	*Amylibacter*	JQ194950.1	98.78%	Uncultured bacterium	NR_146351.1	97.81%	*Amylibacter ulvae*
Otu97	*Arcobacter*	JQ754668.1	99.01%	Uncultured *Arcobacter* sp.	NR_117105.1	98.28%	*Arcobacter ellisii*
Otu98	*Uncultured bacterium*	LR639372.1	98.54%	Uncultured bacterium	NR_114615.1	93.93%	*Nordella oligomobilis*
Otu101	*Flavobacteriaceae_unclassified*	HE979562.1	98.77%	Uncultured bacterium	NR_149768.1	97.79%	*Tenacibaculum sediminilitoris*
Otu103	*Flavobacterium*	LT800200.1	99.27%	Uncultured bacterium	NR_156068.1	98.53%	*Flavobacterium luticocti*
Otu104	*Acinetobacter*	KR072675.1	99.51%	*Acinetobacter* sp. *Lam-1*	NR_165666.1	99.27%	*Acinetobacter chinensis*
Otu106	*Aeromonas*	MT254904.1	99.30%	*Aeromonas enteropelogenes*	NR_116586.1	99.03%	*Aeromonas fluvialis*
Otu108	C39	EU234274.2	98.55%	Uncultured bacterium	NR_028678.1	94.44%	*Azovibrio restrictus*
Otu109	*Fluviicola*	AJ965997.1	98.04%	Uncultured bacterium	NR_112980.1	93.17%	*Salinirepens amamiensis*

Otu110	*Uncultured Cryomorphaceae*	KF917687.1	98.29%	Uncultured bacterium	NR_136475.1	91.48%	*Phaeocystidibacter marisrubri*
Otu114	*Arcobacter*	CP030944.1	98.54%	*Arcobacter aquimarinus*	NR_136421.1	98.54%	*Arcobacter aquimarinus*
Otu117	*Polynucleobacter*	HQ111147.1	98.54%	Uncultured *Polynucleobacter*	NR_151920.1	98.30%	*Polynucleobacter sinensis*
Otu120	*Methylomonas*	HG528975.1	98.79%	Uncultured bacterium	NR_108887.1	98.79%	*Methylomonas paludis*
Otu121	*Polaribacter*	JX304645.1	98.53%	*Polaribacter* sp.	NR_043456.1	98.28%	*Polaribacter dokdonensis DSW-5*
Otu122	*Flavobacterium*	LR650425.1	98.03%	Uncultured bacterium	NR_151882.1	96.56%	*Flavobacterium aquicola*
Otu123	NS3a_marine_group	KU173779.1	98.79%	Uncultured *Bacteroidetes/Chlorobi group bacterium*	NR_025749.1	93.72%	*Maribacter orientalis*
Otu124	*Sediminibacterium*	KP708792.1	98.78%	Uncultured bacterium	NR_133854.1	96.82%	*Seiminibacterium goheungense*
Otu125	*Rheinheimera*	MT833292.1	97.57%	*Pararheinheimera mesophila*	NR_117234.1	97.33%	*Pararheinheimera aquatica*
Otu127	HIMB11	KR077550.1	98.54%	Uncultured bacterium	NR_164623.1	97.32%	*Aliishimia ponticola*
Otu128	*Rheinheimera*	MG576032.1	97.57%	*Pararheinheimera soli*	MG576032.1	97.57%	*Pararheinheimera soli*
Otu132	*Planktomarina*	HE572717.1	100%	Uncultured bacterium	NR_125550.1	99.75%	*Planktomarina temperata*
Otu133	*Microbacteriaceae*_unclassified	CP026923.1	98.55%	*Pontimonas salivibrio*	NR_109611.1	98.31%	*Pontimonas salivibrio*
Otu141	*Uncultured Cryomorphaceae*	KX936675.1	99.27%	Uncultured marine bacterium	NR_132329.1	92.94%	*Phaeocystidibacter luteus*
Otu142	*Pseudohongiella*	MK603568.1	98.54%	Gammaproteobacteria bacterium	NR_126265.1	96.36%	*Pseudohongiella spirulinae*

***Table S16**: A list of reference bacteria species used to construct the reference database*

NR_115936.1_*Vibrio_cholerae*_ATCC14035
NR_029259.1_*Vibrio_mimicus*_1721_77
HQ012017.1_*Salmonella_enterica_subsp._enterica*_DSM17058
NR_026332.1_*Shigella_dysenteriae*_ATCC13313
NR_026331.1_*Shigella_flexneri*_ATCC29903
NR_104901.1_*Shigella_boydii*_P288
NR_104826.1_*Shigella_sonnei*_CECT4887
X80725.1_*E_coli*_ATCC11775
NR_114491.1_*Burkholderia_cepacia*_ATCC25416
NR_029252.1_*Aeromonas_caviae*_ATCC15468
NR_115714.1_*Bacillus_cereus*_CCM2010
NR_116786.1_*Yersinia_enterocolitica_subsp_palearctica*_DSM13030
NR_118147.1_*Mycobacterium_intracellulare_subsp_yongonense*_05_1390
NR_119053.1_*Vibrio_fluvialis*_ATCC33809
NR_043553.1_*Burkholderia_pseudomallei*_ATCC23343
NR_041725.1_*Burkholderia_mallei*_ATCC23344
NR_113629.1_*Burkholderia_gladioli*_strain_NBRC13700
NR_118520.1_*Campylobacter_jejuni_subsp_jejuni*_ATCC33560
NR_118511.1_*Campylobacter_coli*_ATCC33559
NR_118515.1_*Campylobacter_fetus_subsp_venerealis*_NCTC10354
NR_043278.1_*Francisella_tularensis_subsp_novicida*_CIP 56.12
NR_104921.1_*Legionella_pneumophila_subsp_fraseri*_ATCC33156
NR_116040.1_*Mycobacterium_avium_subsp_avium*_ATCC25291
NR_042518.1_*Aeromonas_hydrophila_subsp_ranae*_Au-1D12
NR_119044.1_*Aeromonas_sobria*_ATCC43979
NR_114587.1_*Helicobacter_pylori*_ATCC43504
NR_026078.1_*Pseudomonas_aeruginosa*_DSM50071
NZ_JMTA01000119_*Citrobacter_freundii*_ATCC8090
NR_028894_*Citrobacter_freundii*_ATCC8090
KP326373_*Clostridium_perfringens*_ATCC13124

APPENDIX 4: Curation batchfiles

Sundays Estuary 18S rRNA Mothur batchfile

#make.contigs (file=18SSundays.files, processors=24)

#make.contigs (file=18ST6A1.files, processors=24)

#make.contigs (file=18ST6AR1.files, processors=24)

#make.contigs (file=18ST1ABOT.files, processors=24)

#make.contigs (file=18ST1ARBOT.files, processors=24)

#merge.files (input=18ST6A1.trim.contigs.fasta-18ST6AR1.trim.contigs.fasta, output=M18ST6A1.trim.contigs.fasta)

#merge.files (input=18ST1ABOT.trim.contigs.fasta-18ST1ARBOT.trim.contigs.fasta, output=M18ST1ABOT.trim.contigs.fasta)

#make.group (fasta=M18ST6A1.trim.contigs.fasta-M18ST1ABOT.trim.contigs.fasta, groups=M18ST6A1-M18ST1ABOT)

#merge.files (input=18SSundays.contigs.groups-groups, output=Suns18S.contigs.groups)

#summary.seqs (fasta=Suns18S.trim.contigs.fasta, processors=24)

#screen.seqs (fasta=Suns18S.trim.contigs.fasta, group=Suns18S.contigs.groups, maxambig=0, maxlength=350, minlength=100)

#summary.seqs (fasta=Suns18S.trim.contigs.good.fasta, processors=24)

#unique.seqs (fasta=Suns18S.trim.contigs.good.fasta)

#summary.seqs (fasta=Suns18S.trim.contigs.good.unique.fasta, processors=24)

#count.seqs (name=Suns18S.trim.contigs.good.names, group=Suns18S.contigs.good.groups)

#chimera.vsearch (fasta=Suns18S.trim.contigs.good.unique.fasta, count=Suns18S.trim.contigs.good.count_table, dereplicate=t)

#remove.seqs(fasta=Suns18S.trim.contigs.good.unique.fasta,accnos=Suns18S.trim.contigs.good.unique.denovo.vsearch.accnos)

#summary.seqs (fasta=Suns18S.trim.contigs.good.unique.pick.fasta, count=Suns18S.trim.contigs.good.denovo.vsearch.pick.count_table)

#classify.seqs (fasta=Suns18S.trim.contigs.good.unique.pick.fasta, count=Suns18S.trim.contigs.good.denovo.vsearch.pick.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#remove.lineage (fasta=Suns18S.trim.contigs.good.unique.pick.fasta, count=Suns18S.trim.contigs.good.denovo.vsearch.pick.count_table, taxonomy=Suns18S.trim.contigs.good.unique.pick.nr_v132.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Bacteria)

#summary.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.fasta, count=Suns18S.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#summary.tax(taxonomy=Suns18S.trim.contigs.good.unique.pick.nr_v132.wang.pick.taxonomy, count=Suns18S.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#align.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.fasta, reference=silva.nr_v132.align, flip=T)

#summary.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.align, count=Suns18S.trim.contigs.good
.denovo.vsearch.pick.pick.count_table)

#screen.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.align, count=Suns18S.trim.contigs.good.
denovo.vsearch.pick.pick.count_table, optimize=start-end, criteria=95)

#summary.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.align, count=Suns18S.trim.contigs.good.
denovo.vsearch.pick.pick.good.count_table)

#filter.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.align, vertical=T, trump=.)

#pre.cluster(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.fasta, count=Suns18S.trim.contigs
.good.denovo.vsearch.pick.pick.good.count_table, diffs=2)

#split.abund(count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.count_table, fasta=Suns18S.
trim.contigs.good.unique.pick.pick.good.filter.precluster.fasta, cutoff=1)

#unique.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.fasta, count=Suns18S.
trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.count_table)

#classify.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fasta,
count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table,
reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#remove.lineage(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.fasta,
count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.count_table,
taxonomy=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.nr_v132.wang.pick.tax
onomy, taxon=unknown)

#classify.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.fasta
, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.count_table,
reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#sub.sample(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.fasta,
#count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.count_table,
size=10695, persample=t)

#summary.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.su
bsample.fasta,count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.sub
sample.count_table)

#count.groups(count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.su
bsample.count_table)

classify.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsa
mple.fasta, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.
subsample.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#summary.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.su
bsample.fasta)

#unique.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subs
ample.fasta)

#summary.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fasta, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.count_table)

#dist.seqs(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fasta, cutoff=0.03)

#cluster(column=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.dist, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.count_table, cutoff=0.03, method=furthest)

#make.shared(list=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fn.list, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.count_table, label=0.03)

#rarefaction.single(shared=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fn.shared, calc=sobs, groupmode=F, freq=100)

#classify.otu(list=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fn.list,
count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.count_table, taxonomy=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.nr_v132.wang.taxonomy, label=0.03)

#get.oturep(fasta=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fasta, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.count_table, column=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.dist, list=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fn.list, sorted=name, label=0.03)

#get.seqs(accnos=Suns18S.accnos, fasta=Suns18S.trim.contigs.fasta)

#classify.seqs(fasta=Suns18S.trim.contigs.pick.fasta, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80, count=Suns18S.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.pick.subsample.fn.0.03.rep.count_table)

S**undays Estuary 16S rRNA Mothur batchfile**

#make.contigs(file=SUNS_ALL.files, processors=24)

#make.contigs(file=ST4AR1.files, processors=24)

#make.contigs(file=ST4A1.files, processors=24)

#make.contigs(file=ST5BR1.files, processors=24)

#make.contigs(file=ST5B1.files, processors=24)

#make.contigs(file=ST5B1MRR.files, processors=24)

#make.contigs(file=ST7AR1.files, processors=24)

#make.contigs(file=ST7A1.files, processors=24)

#make.contigs(file=ST7AR0.files, processors=24)

#make.contigs(file=ST7A0.files, processors=24)

#make.contigs(file=ST8BBOT.files, processors=24)

#make.contigs(file=ST8BRBOT.files, processors=24)

#make.contigs(file=ST6A1.files, processors=24)

#make.contigs(file=ST6AR1.files, processors=24)

#merge.files(input=ST7A0.trim.contigs.fasta-ST7AR0.trim.contigs.fasta, output=MST7A0.trim.contigs.fasta)

#merge.files(input=ST7A1.trim.contigs.fasta-ST7AR1.trim.contigs.fasta, output=MST7A1.trim.contigs.fasta)

#merge.files(input=ST5B1.trim.contigs.fasta-ST5BR1.trim.contigs.fasta-ST5B1MRR.trim.contigs.fasta, output=MST5B1.trim.contigs.fasta)

#merge.files(input=ST6A1.trim.contigs.fasta-ST6AR1.trim.contigs.fasta, output=MST6A1.trim.contigs.fasta)

#merge.files(input=ST8BBOT.trim.contigs.fasta-ST8BRBOT.trim.contigs.fasta, output=MST8BBOT.trim.contigs.fasta)

#merge.files(input=ST4A1.trim.contigs.fasta-ST4AR1.trim.contigs.fasta, output=MST4A1.trim.contigs.fasta)

#make.group(fasta=MST7A1.trim.contigs.fasta-MST7A0.trim.contigs.fasta-MST5B1.trim.contigs.fasta-MST4A1.trim.contigs.fasta-MST6A1.trim.contigs.fasta-MST8BBOT.trim.contigs.fasta, groups=MST7A1-MST7A0-MST5B1-MST4A1-MST6A1-MST8BBOT)

#merge.files(input=MST4A1.trim.contigs.fasta-MST7A1.trim.contigs.fasta-MST7A0.trim.contigs.fasta-MST5B1.trim.contigs.fasta-MST6A1.trim.contigs.fasta-MST8BBOT.trim.contigs.fasta-SUNS_ALL.trim.contigs.fasta, output=Suns_All.trim.contigs.fasta)

#merge.files(input=SUNS_ALL.contigs.groups-merge.groups, output=Suns_All.contigs.groups)

#summary.seqs(fasta=Suns_All.trim.contigs.fasta, processors=24)

#screen.seqs(fasta=Suns_All.trim.contigs.fasta, group=Suns_All.contigs.groups, maxambig=0, maxlength=580, minlength=300)

#summary.seqs(fasta=Suns_All.trim.contigs.good.fasta, processors=24)

#unique.seqs(fasta=Suns_All.trim.contigs.good.fasta)

#count.seqs(name=Suns_All.trim.contigs.good.names, group=Suns_All.contigs.good.groups)

#summary.seqs(fasta=Suns_All.trim.contigs.good.unique.fasta, processors=24)

#summary.seqs(count=Suns_All.tim.contigs.good.count_table)

#chimera.vsearch(fasta=Suns_All.trim.contigs.good.unique.fasta, count=Suns_All.trim.contigs.good.count_table, dereplicate=t)

#remove.seqs(fasta=Suns_All.trim.contigs.good.unique.fasta, accnos=Suns_All.trim.contigs.good.unique.denovo.vsearch.accnos)

#summary.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.fasta, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.count_table)

#classify.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.fasta, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#remove.lineage(fasta=Suns_All.trim.contigs.good.unique.pick.fasta, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.count_table, taxonomy=Suns_All.trim.contigs.good.unique.pick.nr_v132.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

#summary.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.fasta, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#count.groups(count=Suns.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#summary.tax(taxonomy=Suns_All.trim.contigs.good.unique.pick.nr_v132.wang.pick.taxonomy, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#align.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.fasta, reference=silva.nr_v132.align, flip=T)

#summary.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.align, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#count.groups(count=Suns.trim.contigs.good.denovo.vsearch.pick.pick.good.count_table)

#screen.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.align, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.pick.count_table, optimize=start-end criteria=95)

#summary.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.align, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.pick.good.count_table)

#filter.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.align, vertical=T, trump=.)

#pre.cluster(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.fasta, count=Suns_All.trim.contigs.good.denovo.vsearch.pick.pick.good.count_table, diffs=2)

#count.groups(count=Suns.trim.contigs.good.unique.pick.pick.good.filter.precluster.count_table)

#split.abund(count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.count_table, fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.fasta, cutoff=1)

#count.groups(count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.count_table)

#sub.sample(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.fasta, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.count_table, size=7800, persample=t)

#unique.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.fasta, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.count_table)

#classify.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fasta, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#dist.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fasta, cutoff=0.03)

#cluster(column=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.dist, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table, cutoff=0.03, method=furthest)

make.shared(list=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fn.list, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table, label=0.03)

#dist.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.fasta, cutoff=0.2, output=lt)

#rarefaction.single(shared=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fn.shared, calc=sobs, groupmode=F, freq=100)

classify.otu(list=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fn.list, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table, taxonomy=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.nr_v132.wang.taxonomy, label=0.03)

#classify.seqs(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.fasta, template=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.count_table)

#get.oturep(fasta=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.fasta, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.count_table, column=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.dist, list=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.fn.list, sorted=name, label=0.03, cutoff=0.03)

#get.seqs(accnos=16SSuns_All.accnos, fasta=Suns_All.trim.contigs.fasta)

#classify.seqs(fasta=Suns_All.trim.contigs.pick.fasta, count=Suns_All.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.subsample.unique.fn.0.03.rep.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

Swartkops Estuary 16S rRNA Mothur batchfile

#make.contigs(file=Swartkops.files, processors=24)

#make.contigs(file=C1A.files, processors=24)

#make.contigs(file=C1AR.files, processors=24)

#make.contigs(file=C4B.files, processors=24)

#make.contigs(file=C4BR.files, processors=24)

#make.contigs(file=C5A.files, processors=24)

#make.contigs(file=C5AR.files, processors=24)

#make.contigs(file=C5B.files, processors=24)

#make.contigs(file=C5BR.files, processors=24)

#make.contigs(file=C7B.files, processors=24)

#make.contigs(file=C7BR.files, processors=24)

#make.contigs(file=C9B.files, processors=24)

#make.contigs(file=C9BR.files, processors=24)

#make.contigs(file=SST3B1.files, processors=24)

#make.contigs(file=SST3BR1.files, processors=24)

#make.contigs(file=SST4A0.files, processors=24)

#make.contigs(file=SST4AR0.files, processors=24)

#make.contigs(file=SST5ABOT.files, processors=24)

#make.contigs(file=SST5ARBOT.files, processors=24)

#make.contigs(file=SST5B0.files, processors=24)

#make.contigs(file=SST5BR0.files, processors=24)

#make.contigs(file=3SW6A.files, processors=24)

#make.contigs(file=3SW6AR.files, processors=24)

#make.contigs(file=3SW6B.files, processors=24)

#make.contigs(file=3SW6BR.files, processors=24)

#merge.files(input=C1A.trim.contigs.fasta-C1AR.trim.contigs.fasta, output=MC1A.trim.contigs.fasta)

#merge.files(input=C4B.trim.contigs.fasta-C4BR.trim.contigs.fasta, output=MC4B.trim.contigs.fasta)

#merge.files(input=C5A.trim.contigs.fasta-C5AR.trim.contigs.fasta, output=MC5A.trim.contigs.fasta)

#merge.files(input=C5B.trim.contigs.fasta-C5BR.trim.contigs.fasta, output=MC5B.trim.contigs.fasta)

#merge.files(input=C7B.trim.contigs.fasta-C7BR.trim.contigs.fasta, output=MC7B.trim.contigs.fasta)

#merge.files(input=C9B.trim.contigs.fasta-C9BR.trim.contigs.fasta, output=MC9B.trim.contigs.fasta)

#merge.files(input=SST3B1.trim.contigs.fasta-SST3BR1.trim.contigs.fasta, output=MSST3B1.trim.contigs.fasta)

#merge.files(input=SST4A0.trim.contigs.fasta-SST4AR0.trim.contigs.fasta, output=MSST4A0.trim.contigs.fasta)

#merge.files(input=SST5ABOT.trim.contigs.fasta-SST5ARBOT.trim.contigs.fasta,
output=MSST5ABOT.trim.contigs.fasta)

#merge.files(input=SST5B0.trim.contigs.fasta-SST5BR0.trim.contigs.fasta, output=MSST5B0.trim.contigs.fasta)

#merge.files(input=3SW6A.trim.contigs.fasta-3SW6AR.trim.contigs.fasta, output=M3SW6A.trim.contigs.fasta)

#merge.files(input=3SW6B.trim.contigs.fasta-3SW6BR.trim.contigs.fasta, output=M3SW6B.trim.contigs.fasta)

#merge.files(input=MC1A.trim.contigs.fasta-MC4B.trim.contigs.fasta-MC5A.trim.contigs.fasta-MC5B.trim.contigs.fasta-MC7B.trim.contigs.fasta-MC9B.trim.contigs.fasta-MSST3B1.trim.contigs.fasta-MSST4A0.trim.contigs.fasta-MSST5ABOT.trim.contigs.fasta-MSST5B0.trim.contigs.fasta-M3SW6A.trim.contigs.fasta-M3SW6B.trim.contigs.fasta-Swartkops.trim.contigs.fasta, output=Swart.trim.contigs.fasta)

#make.group(fasta=MC1A.trim.contigs.fasta-MC4B.trim.contigs.fasta-MC5A.trim.contigs.fasta-MC5B.trim.contigs.fasta-MC7B.trim.contigs.fasta-MC9B.trim.contigs.fasta-MSST3B1.trim.contigs.fasta-MSST4A0.trim.contigs.fasta-MSST5ABOT.trim.contigs.fasta-MSST5B0.trim.contigs.fasta-M3SW6A.trim.contigs.fasta-M3SW6B.trim.contigs.fasta, groups=MC1A-MC4B-MC5A-MC5B-MC7B-MC9BR-MSST3B1-MSST4A0-MSST5ABOT-MSST5B0-M3SW6A-M3SW6B)

#merge.files(input=Swartkops.contigs.groups-merge.groups, output=Swart.contigs.groups)

#summary.seqs(fasta=Swart.trim.contigs.fasta, processors=24)

#screen.seqs(fasta=Swart.trim.contigs.fasta, group=Swart.contigs.groups, maxambig=0, maxlength=580, minlength=350)

#summary.seqs(fasta=Swart.trim.contigs.good.fasta, processors=24)

#unique.seqs(fasta=Swart.trim.contigs.good.fasta)

#summary.seqs(fasta=Swart.trim.contigs.good.unique.fasta, processors=24)

#count.seqs(name=Swart.trim.contigs.good.names, group=Swart.contigs.good.groups)

#chimera.vsearch(fasta=Swart.trim.contigs.good.unique.fasta, count=Swart.trim.contigs.good.count_table, dereplicate=t)

#remove.seqs(fasta=Swart.trim.contigs.good.unique.fasta,accnos=Swart.trim.contigs.good.unique.denovo.vsearch.accnos)

#summary.seqs(fasta=Swart.trim.contigs.good.unique.pick.fasta, count=Swart.trim.contigs.good.denovo.vsearch.pick.count_table)

#classify.seqs(fasta=Swart.trim.contigs.good.unique.pick.fasta, count=Swart.trim.contigs.good.denovo.vsearch.pick.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#remove.lineage(fasta=Swart.trim.contigs.good.unique.pick.fasta, count=Swart.trim.contigs.good.denovo.vsearch.pick.count_table, taxonomy=Swart.trim.contigs.good.unique.pick.nr_v132.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

#summary.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.fasta, count=Swart.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#summary.tax(taxonomy=Swart.trim.contigs.good.unique.pick.nr_v132.wang.pick.taxonomy, count=Swart.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#align.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.fasta, reference=silva.nr_v132.align, flip=T)

#summary.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.align, count=Swart.trim.contigs.good.denovo.vsearch.pick.pick.count_table)

#screen.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.align, count=Swart.trim.contigs.good.denovo.vsearch.pick.pick.count_table, optimize=start-end, criteria=95)

#filter.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.align, vertical=T, trump=.)

#split.abund(count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.count_table, fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.fasta, cutoff=1)

#count.groups(count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.count_table)

#unique.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.count_table)

#classify.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#remove.lineage(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.count_table, taxonomy=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.nr_v132.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

#count.groups(count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.count_table)

#sub.sample(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.count_table, size=8497, persample=t)

#summary.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table)

#count.groups(count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table)

#classify.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)

#dist.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fasta, cutoff=0.03)

#dist.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.fasta, cutoff=0.03)

#cluster(column=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.dist, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table, cutoff=0.03, method=furthest)

#make.shared(list=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fn.list, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table, label=0.03)

#rarefaction.single(shared=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fn.shared, calc=sobs, groupmode=F, freq=100)

#classify.otu(list=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fn.list, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_

table, taxonomy=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.nr_v132.wang.taxonomy, label=0.03)

#classify.seqs(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fasta, template=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table)

#get.oturep(fasta=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.count_table, list=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fn.list, column=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.dist, sorted=name, label=0.03)

#get.seqs(accnos=16s_AllSwarts.accnos, fasta=Swart.trim.contigs.fasta)

#classify.seqs(fasta=Swart.trim.contigs.pick.fasta, count=Swart.trim.contigs.good.unique.pick.pick.good.filter.precluster.abund.unique.pick.subsample.fn.0.03.rep.count_table, reference=silva.nr_v132.align, taxonomy=silva.nr_v132.tax, cutoff=80)