Physical, chemical and biological processes and fates ofpetroleum-based plastic and bioplastic pollutants in aquatic environments

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

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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List of Abbreviations

^{o}C	Degrees Celsius
µ	Micro (10^{-6})
%	Percent
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
ASE	Accelerated Solvent Extraction
ASTM	American Society for Testing and Materials
bp	Base pair
cm	Centimetre
d	Day
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DO	Dissolved oxygen
et al.	"and others"
EC	Electrical conductivity
FTIR	Fourier transform infrared spectroscopy
g	Gram
G	Giga (10^{9})
GC	Gas chromatography
GPC	Gel permeation chromatography
h	Hour
HOC	Hydrophobic organic chemicals
IDL	Instrument detection limit
IMO	International Marine Organisation
IQL	Instrument quantitation limit
kb	Kilobase pair (10^{3}basepairs)
K_{ow}	Water octanol partition coefficient
L	Litre
LCS	Laboratory control sample
LOD	Limit of detection
lw	Lipid weight
m	Metre
m (as a prefix)	Milli (10^{-3})
M	Molar
MDL	Method detection limit
min	Minute
mol	Mole
MQL	Method quantitation limit
MS	Mass spectrometer
n	Number of replicates
N	Newton
NBFR	Novel brominated flame retardants
N.D	Not detected
nMDS	Non-Metric Multidimensional Scaling

OTU	Operational taxonomic unit
PBDE	Polybrominated diphenyl ether
PC	Polycarbonate
PCR	Polymerase chain reaction
PE	Polyethylene
PET	Poly(ethylene terephthalate)
PERMANOVA	Permutational Multivariate Analysis of Variance
pH	Measure of acidity or alkalinity of a solution
PHA	Poly(hydroxyalkanoates)
PLA	Polylactic acid
POD	Photo-oxidative degradation
PP	Polypropylene
PS	Polystyrene
PVC	Poly(vinyl chloride)
QA	Quality Assurance
QC	Quality Control
rRNA	Ribosomal ribonucleic acid
ROP	Ring-opening polymerisation
S	Svedberg unit
S/N	Signal to noise ratio
S-PLE	Selective pressurised liquid extraction
SEM	Scanning electron microscope
SIMPER	Similarity percentage
SSU	Small subunit
UV-B	Ultraviolet light (280–320 nmwavelength)
UN	United Nations
W	Watt
WCA	Water contact angle

Executive Summary

By the mid to late 20th Century the use of petroleum-based plastics had become widespread. Much of this plastic has been and continues to be littered, leading to plastic pollution becoming ubiquitous in marine environments. Plastic pollutants can cause physical harm to marine organisms, via entanglement or ingestion. Persistent organic pollutants (POPs) such as some brominated flame retardants (BFRs) are known to accumulate onto plastic pollutant surfaces in marine environments, and may provide a novel pathway for exposure to these chemicals to organisms. Additionally, plastic surfaces containing microbial biofilms have been suggested as a vector for the transport of harmful algae and pathogens beyond their natural ranges. In recent decades, bioplastics(plastics derived from biological based materials) have been developed and utilised as an alternative to petroleum-based plastics. However, the environmental fates of bioplastic pollutants, the processes of BFR accumulation and biofilm development onto bioplastics remainsundetermined.

This thesis sought to advance current knowledge of the fate of pollutant petroleum-based plastics and bioplastics within aquatic ecosystems.This was addressed via an experimental approach in which polypropylene (PP) as a model petroleum-based plastic, polylactic acid (PLA) as a model bioplastic and glass slides as non-plastic control substratewere deployed in anexposure experiment at five sites along a freshwater-marine continuum of the Yarra River into Port Phillip Bay, Melbourne, Australia. The three specific objectives were to; compare variation in the structural properties of PP and PLA, via analysis of surface hydrophobicity, tensile strength, crystallinity and chemical structure (Chapter 3); determine the potential for BFRs to accumulate onto PP and PLA with comparison to glass substrates (Chapter 4); and compare spatial-, temporal- and substrate-specific (PP, PLA and glass) variation in the structure and composition of microbial (prokaryotic and eukaryotic) biofilm communities

forming on polymer and glass surfaces and with comparison to those in the surrounding water (Chapter 5).

Research in Chapter 3 revealed that between Day 1 and Month 12 of the exposure experiment there were no significant changes in either the water contact angle (WCA) (a proxy used to assess surface hydrophobicity), or Young's Modulus (a measure related to tensile strength) for either PP and PLA substrates. There was no overall trend of an increase of the Max load at break (Max Load) (a measure related to tensile strength) of the PLA substrates between the other sampling dates. However, there was a significant increase ($P < 0.05$) of the Max Load for the PLA substrates but not the PP substrates. Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) (crystallinity) analysis of the plastics were undertaken to compare polymers between Day 1 and Month 6. There was an increase in the crystallinity of the PLA substrates but not for the PP substrates. Neither the PP nor the PLA substrates exhibited any change in FTIR spectra between Day 1 and Month 6 and indicated no change in the chemical structure of either plastic type.

Research in Chapter 4 investigated accumulation of two groups of BFRs onto the PP, PLA and glass substrates. The targeted BFRs were polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs). The selected BFR analytes were extracted from the PP and glass substrates using a selective pressurised liquid extraction (S-PLE)method. A novel dual vortex and sonication method was successfully developed and implemented for the extraction of BFR analytes from the PLA substrates. Analysis of the selected BFRs was undertaken using an Agilent 7000C gas chromatograph coupled to a triple quadrupole mass spectrometer (GC-MS/MS). Differences in BFR concentrations between the three substrate types was not able to be assessed due to sample loss from sample frames over the course of the experiment. At least one PBDE congener and one NBFR compound were detected in all

samples, although the mean ∑PBDE and ∑NBFR concentrations on the substrates were low, 12.3 ng g^{-1} ± 7.4 ng g^{-1} and 23 ng g^{-1} ± 23 ng g^{-1}, respectively.

Research in Chapter 5 explored structural and compositional changesin the prokaryotic and eukaryotic microbial biofilm communities as well as water communitiesvia high-throughput DNA amplicon sequencing of the 16S and 18S rRNA genes, respectively. The structure of the microbial biofilm communities on substrates were distinct from those in the surrounding water environment and differed principally with sample site, and then with sampling date. There was no significant ($P > 0.05$) difference in the composition of microbial biofilm communities between any of the three substrate types. The prokaryotic biofilms communities were dominated by Proteobacteria (alpha-, beta-, and gamma- classes) and Bacteroidetes, and the eukaryotic biofilm communities weredominated by diatoms and ciliates. Both the prokaryotic and eukaryotic water microbial community types had higher mean numbers of observed operational taxonomic units (OTUs) and Shannon diversity when compared to the coupon-biofilm communities. The relative abundance of three key functional guilds of potential plastic degraders, pathogenic bacteria and harmful algae were assessed. None of these three functional guilds had relative abundancesgreater than 1 % of the overall community;although three fish pathogens *Pseudomonas anguilliseptica*,*Acinetobacter johnsonii* and *A. lwoffii*, were frequently identified, being detected in over two thirds of the biofilm communities.

This research has shown thatPLA is as physically- and chemically-stable as PP over a 12-month period in aquatic environments. It was hypothesised that substantial degradation of the plastics did not occur because the plastics were quickly biofouled within days from deployment, and that biofouling in water would have reduced the rate of photo-oxidative degradation, one of the main degradation processes to occur to plastics in natural environments.PLA, PP and glass substrates wereall found to have the capability to accumulate

PBDEs and NBFRs. The plastic biofilm communities were shown to be diverse and distinct from those in the surrounding water communities, and the plastic biofilm (both PP and PLA) communities were highly similar to those forming on glass, demonstrating that plastic biofilm communities consists of predominantly generalist surface colonisers. Three fish pathogens (*P. anguilliseptica*, *A. johnsonii* and *A. lwoffii*) were frequently identified within the substrate biofilm communities, indicating that aquatic plastic debris may be a long-term novel exposure pathway for pathogen exposure in fish due to the high number of plastic fragments in aquatic environments, and the ability of plastics to passively travel vast distances. The lack of plastic degrading organisms identified on the plastics raises doubts that PLA, will be biodegraded to any significant extent in aquatic environments.Therefore, bioplastics should be held in the same regard as petroleum-based plastics by government, policy makers and industry leaders as they work towards solutions that reduce the impacts of both petroleum-based plastics and bioplastics within aquatic ecosystems.

Conference Proceedings

Oral presentation presented at “Micro2016” May 2016, Lanzarote, Spain, entitled as “Fates of plastic pollution in a major urban river: persistence and bacterial colonisation of oil-based plastics and bioplastics in the Yarra River, Melbourne, Australia” by **Taylor Gundry,** Slobodanka Stojkovic, Mark Osborn.

Chapter 1: Introduction

1.1 Plastic productionand applications

Global plastic production in the last 50 years has increased substantially,from an estimated 1.7 million tonnes per annum to 380 million tonnes per annumin 2015 (Geyer *et al.*, 2017). Plasticsare widely used in packaging (<30%), building, manufacturing, automotive, aerospace, electrical, electronic and medical industries (PlasticsEurope, 2012). They have been widely used by industry because they have a wide range of beneficial characterises which include being lightweight, durable and strong and they are considered bio-inert, chemically stable and economical to produce compared to traditional materials such as wood, metal and glass(Andrady, 2011). For instance, plastic packaging can extendthe shelf-life of food by maintaining a barrier around the food item that is impenetrable to microorganisms, moisture or air. For example, in the Netherlands,the introduction of modified atmosphere plastic packaging, resulted in an annual decrease of 11,000 tons of spoilt meat from supermarket shelves (Thoden van Velzen and Linnemann, 2008). Environmental benefits of using plastic in place of other materials extend to the building industry. The use of plastic-based insulation batts has a greenhouse gas emissions recovery time after only four months of use by reducing the building demand for heating and cooling; and are estimated to save 150 more times the energy costs to produce them over the life-cycle of the insulation batts (Pilz and Mátra, 2006).

The first synthetic plastic produced on a commercial scale was Bakelite, developed by the Belgian chemist Leo Baekeland in 1907 (Baekeland, 1907). It was a thermoset plastic; meaning once it has set it cannot be remelted or remoulded.During manufacturing, the individual polymers of thermoset plastics form cross-links via strong covalent chemical bonding, creating a rigid structure.This is opposed to thermoplastics which do not form cross-

links and can be remelted once set and remoulded, and therefore can be recycled (Ensinger, 2014). Bakelite was used extensively in the electrical and automobile industries due to its high heat and electrical resistance (American Chemical Society, 2014). As the plastic industry grew in the early 20th Century, more types of plastics were created, this included the development of thermoplastics(Table 1.1). The production of thermoplastics soon overtook the production of thermosets so that now, in the U.S, production of thermoplastics is approximately six times higher than that of thermoset plastics (American Chemical Council, 2013).

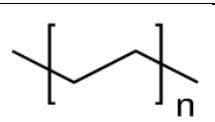
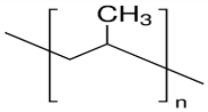
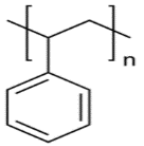
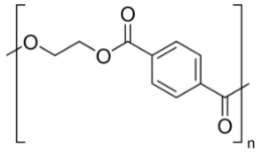
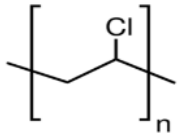
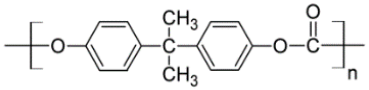
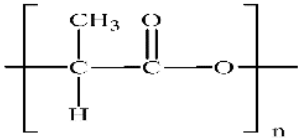
Table 1.1Timeline of plastic development.

Significant year of development*	**Plastic type**	**Reference**
1907	Bakelite	(Baekeland, 1907)
1913	Cellophane	(Berl, 1913)
1933	Poly(vinyl chloride)	(Semon, 1933)
1937	Polyethylene	(Perrin *et al.*, 1937)
1935	Polyamide (Nylon)	(Carothers, 1938)
1941	Poly(ethylene terephthalate)	(Whinfield and Dickerson, 1949)
1953	Polypropylene	(Hogan and Banks, 1958)
1953	Polycarbonate	(Schnell *et al.*, 1962)
1987	Polyhydroxybutyrate (PHB) (BioPol®)	(Westlake, 1987)
1994	Polylactic acid (PLA)	(Gruber and O'Brien, 2005)

* Year of significance indicating when the polymer was either discovered, a patent was awarded or commercial production begun.

Of the 20 different classesof plastic produced (Thompson *et al.*, 2004), only fiveclassescomprise approximately 80% of the total plastic production in 2011; these were polyethylene (PE), polypropylene (PP), polystyrene (PS), polystyrene (PS), poly(ethylene terephthalate) (PET) and poly(vinyl chloride) (PVC) (Andrady, 2011). Common plastics that are produced are shown in Table 1.2. Of the top five plastics produced, only two have molecular densities greater than water, these are PVC and PET. Therefore, most plastics are highly buoyant and mobile in aquatic environments.

Table 1.2Common plastics and bioplastics densities, applications and chemical structures.

Plastic type	**Abbreviation**	**Density (g cm^{-3})**	**Applications**	**Chemical Structure**	**Polymer Type**
Low density polyethylene	LDPE	0.91-0.93	Carrier bags, squeeze bottles		Thermoplastic
High density polyethylene	HDPE	0.94-0.96	Milk Bottles, motor oil containers		Thermoplastic
Polypropylene	PP	0.89-0.91	Bottle Caps, food containers		Thermoplastic
Expanded polystyrene	EPS	0.02-0.06	Shock absorbers in packaging		Thermoplastic
Poly(ethylene terephthalate)	PET	1.38-1.40	Drink bottles, fibres		Thermoplastic
Poly(vinyl chloride)	PVC	1.20-1.55	Plumbing pipes, shower curtains, chemical resistant gloves		Thermoset plastic
Polycarbonate	PC	1.36	Microwavable plastic containers, baby bottles		Thermoplastic
Polylactic acid	PLA	1.25	Food containers		Bioplastic

1.2 Production and life-cycle assessments of bioplastics

More recently, with the aim of reducing fossil fuel use to create plastics, biological-based plastics have been designed and are now being increasingly used (European Bioplastics, 2013).In 2017, theglobal production of bioplastics was approximately 2.05 million tonnes per annum, and is modelled to increase to 2.44 million tonnes per annum by 2022 (European Bioplastics, 2017). The two bioplastic polymer types that are of the main contenders for future large-scale production and usage as bioplastics are polylactic acid (PLA) and poly(hydroxyalkanoates) (PHA) (European Bioplastics, 2017). PLA is mainly produced using plant starches from corn kernels(Vink *et al.*, 2003). However, due to its weak thermal stability properties(Nguyen *et al.*, 2018), PLA is unsuitable for many applications that involve high heat (Sudesh and Iwata, 2008). PHAs are produced naturally by many bacteria (Wallen and Rohwedder, 1974), and can also be used as a carbon and energy source for microbial growth(Steinbüchel and Schlegel, 1991). A wide range of microorganisms including*Ralstonia eutropha*(*Alcaligenes eutrophus*), *Pseudomonas aeruginosa* and *Bacillus megaterium*are able to synthesise PHAs (Lee, 1996, Reddy *et al.*, 2003). Recombinant bacteria are used commercially to synthesise PHAs; however their synthetic yields remain low (Keshavarz and Roy, 2010), which lead to high production costs that are as much as 10 times higher than those of traditional petroleum-based plastics (Kosseva and Rusbandi, 2018). PHAs currently account for only 2.4% of global bioplastic production (European Bioplastics, 2017).

A report by CargillDow (the largest producer of PLA), concluded that the production of PLA uses 25 % - 55% less fossil fuels than petroleum-based plastics because the main feedstock of PLA is from a renewable resource (corn starches)(Vink *et al.*, 2004). However, the authors appear to not have allowed for the cost of growing and processing the corn. Conversely, another life-cycle assessment of PLA and PET bottles concluded that the impacts of both polymer types were highly similar (Gironi and Piemonte, 2011); this was primarily due to the

associated environmental costs of growing the corn used to produce the PLA such as the fuel used by farm machinery, increased demand for water resources and the diversion of arable land from food production. Therefore,there may be little direct environmental benefit from using bioplastics when compared to traditional petroleum-based plastics.

1.3 Plastic pollution in aquatic environments

Plastic pollution is a global problem, with an estimated 15 to 51 trillion particles floating on the ocean surface (van Sebille *et al.*, 2015). These plastic pollutants can harm marine organisms from zooplankton (Cole *et al.*, 2013)to seabirds (Lavers *et al.*, 2014).This harm can be caused by either physical damage and/or exposure to toxic persistent organic pollutants (POPs)(Teuten *et al.*, 2009) that have been found to adsorb and concentrate onto plastic surfaces from the surrounding aquatic environment (Ogata *et al.*, 2009), and transfer to organisms once ingested (Chua *et al.*, 2014, Wardrop *et al.*, 2016). Plastic pollutants also offer a scaffold for many marine organisms to adhere to, from bacteria to algae and invertebrates (Reisser *et al.*, 2014). There is a potential for plastic pollutants to act as rafts and transport marine pathogens far outside their natural domain (Carson *et al.*, 2013), and thereby pose another potential threat to marine ecosystems. In addition, it is estimated that plastic pollution causes a natural capital cost to the environment of $13 billion per annum due to losses sustained by fisheries and tourism industries and the costs associated with cleaning beaches (United Nations Environment Programme, 2014).

1.4 Plastic pollution distributionin the environment

1.4.1 Sources and pathways of plastic intoaquatic environments

Plastic pollution in the marine environment was first reported in the early 1970s (Carpenter *et al.*, 1972, Carpenter and Smith Jr, 1972). Plastic debris was found in the Sargasso Sea at a concentration of 3,500 particles km^{-2}, most of which had diverse communities of diatoms and hydroids attached(Carpenter and Smith Jr, 1972). Since then plastic debris has been found in some of the most remotest places on the planet, from the Southern Ocean (Barnes *et al.*, 2010) to Artic sea ice (Obbard *et al.*, 2014) where they were not likely disposed, demonstrating that plastic pollution has the potential to travel large distances and also to be extremely recalcitrant

in the environment.The ocean is believed to be the major sink for plastic debris, other than in municipal waste streams (Browne *et al.*, 2011).

Urban centres and the global fishing industry are major continuous sources of plastic debris through the process of littering (Donohue *et al.*, 2001, Edyvane *et al.*, 2004, Eriksen *et al.*, 2013, Horton *et al.*, 2017). Plastic debris in urban environments are frequently washed into stormwater systems after rain events, which then help transport the plastic debris out into aquatic environments (Melbourne Parks and Waterways, 1993). Sewage treatment outflow is another pathway for transport of plastics from urban environmentsinto aquatic environments (Browne *et al.*, 2010, Browne *et al.*, 2011). Browne et al. (2011) found that the average sewage effluent discharge from a tertiary-level water treatment plant based in New South Wales (Australia) contained one particle of microplastic per litre, with polyester comprising 67% of the debris and acrylic 17%. These plastic fibres are likely entering sewage systems via the use of washing machines; one washing load (6 kg acrylic fabric) has been estimated to release over 700,000 fibres (Napper and Thompson, 2016). Natural disasters such as tsunamis can act as large point source events (Lebreton and Borrero, 2013). For example the 2011 Tohoku tsunami caused an estimated 1,000 times more plastic to enter the Pacific Ocean than the normal litter rate for the east coast of Japan (Lebreton and Borrero, 2013).

1.4.2 Distribution of plastic in freshwater systems

Rivers are the main conduit for plastic transport into the oceans.Understanding the amount of plastic they can transport is extremely important when trying to determine the ultimate fate and impact of plastic debris in the oceans (Rech *et al.*, 2014). Estimating thetotal volume of plastic in rivers is difficult as rivers are constantly in flux, and are highly influenced by factors such as the surrounding catchments and seasonal variation in flow(Lebreton *et al.*, 2017). A two year study of plastic abundance, by volume, in the Austrian section of the River Danube

by Lechner *et al.* (2014) found the mean plastic abundance was 318.8 ± 4,664.6 items per 1,000 m^{-3}. The large standard deviation also highlights the heterogeneous variability of plastic distribution, 79.4% of the debris consisted of industrial pre-production pellets. The authors calculated these overall plastic debris load from the Danube into the Black Sea was 4.2tonnes per day, this equates to 1,533 tonnes per year. This value is more than the estimated total plastic load in the North Atlantic sub-tropical gyre as proposed by Law *et al.* (2010). Furthermore, the authors suggest that the results may be an underestimate due to several factors including; microplastics flowing through the net pores, items >5 cm being diverted from the net mouth and countries downstream of Austria contributing more plastic debris due to lower standards of wastewater management. In the U.S.A, based on 142 one litre water samples, it has been estimated that an average of 300 million plastic fibres flow from the Hudson River, New York, into the Atlantic Ocean per day, and the Patapsco River, Maryland, had an estimated 297,927 ± 180,252 plastic pieces km^{-2}(Yonkos *et al.,* 2014).Yet the area of plastic debris loads in rivers remains under researched and highly skewed towards European and North American countries. For instance, a meta-analysis study that sought to build a predictive model of plastic inputs into the ocean from rivers relied on observational studies (as well as factors such as waste mismanagement rate and annual runoff) from only 13 rivers, only one of which was in Asia (the Yangtze River), and none of which were from Africa (Lebreton *et al.,* 2017).Lebreton *et al.* (2017)concluded that between 1.15 Mt and 2.41 Mt of plastic debris per year enters the oceans via rivers, 67 % of whichis from Asian countries.

A study conducted by Morritt *et al.* (2014) monitored the abundance of plastic debris in the River Thames, UK. The study was intended to monitor the abundance of invasive Chinese mitten crabs(*Eriocheir sinensis*),and the study of plastic debris in the river was added retrospectively. As the fyke nets were placed 40cm from the river bed in order to catch the crabs, an insightful look into the distribution of the river's water column was observed. The

researchers were able to collect 8,490 pieces of submerged plastic over a 3 month period. Interestingly sanitary products accounted for approximately 20% of the debris collected. The most likely source of these products is through sewerage outflows suggesting many consumers are not disposing of sanitary products correctly. This was an important finding as most estimates of plastic abundance in aquatic systems use surface trawl methods to collect the plastic and therefore do not take into account the three dimensional spatial properties of plastic pollution in the water column.

The abundance of plastic debris in lakes is currently not well understood with a limited amount of research having been published. Three studies into the abundance of plastic debris in the Great Lakes of North America (Zbyszewski and Corcoran, 2011, Eriksen *et al.*, 2013, Zbyszewski *et al.*, 2014); found that the abundance of plastic in the Great Lakes was in the order of 2.8–105 $x10^3$particles km^{-2}. Conversely the abundance of plastic debris in the Three Gorges Dam in China is reported to be on average 8.56 x 10^6 particles km^{-2}(Zhang *et al.*, 2015a).

1.4.3 Distribution and abundance of plastic in the marine environment

Researchers are beginning to try and estimate the total load of plastic debris in the world's oceans(Cozar *et al.*, 2014, Eriksen *et al.*, 2014, van Sebille *et al.*, 2015).Cozar *et al.* (2014)estimated the total amount of plastic debris in the world's oceans to be between6,350 and 31,751 metric tons,Eriksen *et al.* (2014)estimated a minimum of 5.25 trillion particles in surface waters, weighing 2,439,778 metric tons and thevan Sebille *et al.* (2015)modelpredicted a minimum of 15 trillion particles of plastic weighing between 93,000 and 236,000 metric tons. This large increase compared from the Cozar *et al.* (2014)study andEriksen *et al.* (2014) studies to the van Sebille *et al.* (2015) is partially due to the van Sebille *et al.* (2015) study incorporating the mixing effect of wind on ocean surface plastic.

All of these models require large assumptions, therefore the relative abundance instead of absolute abundancesof plastic debris between locations is currently more useful (Ronan, 2017).

Eriksen *et al.* (2014)found that microplastics were the dominant fraction by number of particles, with some concentrated areas in the oceanic gyres having over 500,000 microplastic particles per km^2. However, Cozar *et al.* (2014)found that microplastics in the range of 0.5 mm to 5mm were lower in abundance than was predicted by modelling by several orders of magnitude;whereas the abundance of larger sizes of plastic debris (>5 mm) were consistant with the models. The findings by Cozar *et al.* (2014) suggests that there is a size selective sink for plastics beyond the ocean surface for microplastics. One of the possible sinks is from the consumption of the microplastics in this size range by zooplanktivorious fish (Cozar *et al.*, 2014). The plastic may also be sinking from the ocean surface to greater depths due to biofouling which can increase the density of the plastic fragment(Cozar *et al.*, 2014), and may be why the mesopelagic fish discussed earlier are consuming plastic (Davison and Asch, 2011, Choy and Drazen, 2013). However,it is unknown the volume of this plastic that is reaching the seafloor due to a number of factors. These factors include that there is a positive relationship between depth and seawater density, so plastics will reach a neutral density at different depths, as well as the difficulty of obtaining samples from remote and deep locations due to the technical and monetary resources required.

Benthic grabs have been the main technique usually implemented for surveys of benthic plastic debris, this techniqueis not recommended for sampling such a heterogeneously spread pollutant because they cover small and discrete patches of seabed(Barnes *et al.*, 2010, Ling *et al.*, 2017). Also, most research into plastic debris in ocean sediments hasoccurredin coastal metropolitan waters (Hanvey *et al.*, 2017), plastic abundance correlating to proximity to urban centres (Browne *et al.*, 2010). However, plastics have still been found to be ubiquitous in

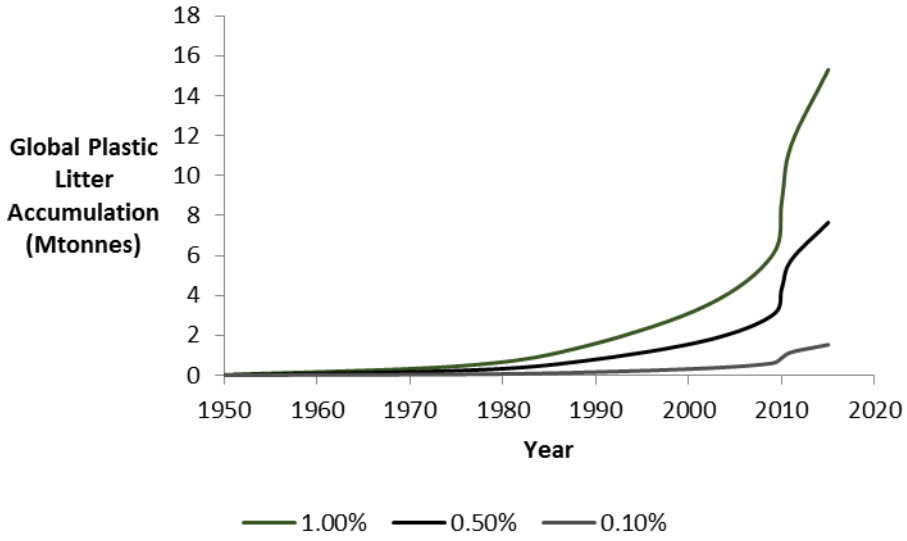
remote marine sediments, Ling *et al.* (2017)found 9,552 microplastics from only 42 sediment samples (a total of 2.84 L) from the south-east coast of Australia.Also, Chiba *et al.* (2018)observed plastic debris at a depth of 10,898 m in the Mariana Trench using a deep-sea submersible, with a total of 3,425 anthropogenic debris (the largest proportion of which was plastic (33 %)) being observed during 5,010 dives in the Pacific, Atlantic and Indian oceans.

The research on plastic pollution abundance in the oceans byCozar *et al.* (2014), Eriksen *et al.* (2014) and van Sebille *et al.* (2015) are all limited by the lack of data sourced from non-Western countries such as from Asian countries, especially from India and China. This is a severe limitation when trying to determine the amount of plastic debris in the ocean surfaces, as they are regions of very high human populations and frequently have poor waste management practices (Jambeck *et al.*, 2017). However, since these meta-analyses were published, articles documenting plastic pollution in previously undocumented marine waters have been published from South Africa, China, Japan and Pakistan (Isobe *et al.*, 2015, Zhou *et al.*, 2016, Fok and Cheung, 2015, Ali and Shams, 2015, Nel and Froneman, 2015). Plastic pollution in the Sea of Japan was 1,720,000 pieces km^{-2}(Isobe *et al.*, 2015), 3.4 times greater than in the North Pacific Subtropical Gyre (500,000 pieces km^{-2}; Law *et al.* (2014)), a key area for the Cozar *et al.* (2014)andEriksen *et al.* (2014) meta-analyses. Therefore, estimates of the total amount of plastic in the oceans may be grossly lower than the true value. Plastic pollutants in Australian coastal waters are considerably lower than elsewhere in the Indian and Pacific oceans with a mean concentration of 4,256 pieces km^{-2}(Reisser *et al.*, 2013). Most of the plastic was concentrated around metropolitan areas of Sydney and Brisbane, it should be noted that data from Port Phillip Bay (Melbourne) was not collected(Reisser *et al.*, 2013).

Plastic debris along coasts has been well documented and researched with many shore-based plastic abundance surveys having been undertaken along beachesaround the world in places including Antarctica (Eriksson *et al.*, 2013), Chile (Thiel *et al.*, 2013), the Falkland Islands

(Otley and Ingham, 2003), Germany (Liebezeit and Dubaish, 2012), Indonesia (Uneputty and Evans, 1997)Singapore (Ng and Obbard, 2006), South Korea (Hong *et al.*, 2014) and the USA (Cutter *et al.*, 1991, Gilligan *et al.*, 1992, Donohue *et al.*, 2001). There is difficulty in attempting to quantify the amount of plastic debris along a given beach, and then being able to compare it to other studies. This is for several reasons; primarily it is often unknown or unreported how long the debris has been accumulatingand secondarily different units for reporting and sampling techniquesare often employed.

The rate of plastic accumulation into the oceans from rivers is estimated to be between 1.15 and 2.41million tonnes per year, 67% of which is generated in Asian countries(Lebreton *et al.*, 2017),while an estimated 4.8-12.7 million tonnes per year of plastic in total is estimated to flow in the oceans from coastal populations (Jambeck *et al.*, 2015), which is about 1.7% to 4.6% of total plastic production (Geyer *et al.*, 2017).Jambeck *et al.* (2015)also factored in plastic inputs such as direct littering on beaches and the inputs from urban stormwater systems. Figure 1.1is an estimation of the global plastic litter accumulation in marine environments based on a range of three constant litter rates (1.0%, 0.5% and 0.1%) of global plastic production estimates produced by PlasticsEurope (2012)and Geyer *et al.* (2017).While the production of plastic is linear in nature,since the amount of plastic produced in the previous year has little effect on the production of the current year; while the accumulation of plastic debris in the environment due to its slow rate of degradation is exponential in nature, with the amount of plastic in the environment building on the previous year's accumulated debris(Vannela, 2012).



1) $x_a = (P_i \times LR_i)$

2) $x_b = x_a + (P_{ii} \times LR_{ii})$

Figure 1.1Hypothesised global buoyant plastic litter accumulation (M tonnes) in the marine environment. The litter rate is based on 1.0%, 0.5% and 0.1% of the production for the corresponding year. The model assumes that all plastics that have entered the marine environment are buoyant and remain present. The production statistics are based on production estimates by PlasticsEurope (2012) and Geyer *et al.* (2017). Equations, P = Annual global plastic production (Mtonnes) LR= Litter rate percentage, *i* = initial values, *ii*= second set of values.

1.5 Degradation pathways of plastic debris

1.5.1 Abiotic degradation pathways of plastic

Petroleum-based plastics are extremely recalcitrant in aquatic environments, with expected half lives of hundreds of years (Barnes *et al.*, 2009). Physical degradation of plastics by the processes of abrasion and shearing are the main processes for plastic degradation in aquatic environments (Barnes *et al.*, 2009). As plastics fragments mechanically, the chemical structure of these plastics remains the same. The complete mineralisation of plastics is an extremely slow process (Andrady, 2011).The main chemical degradation pathways are photo-oxidative degradation (POD), hydrolysis andthermal degradation, as well as biological degradation(Goldberg, 1995, Olayan *et al.*, 1996, Shah *et al.*, 2008); although thermal degradation of plastics does not normally occur under natural conditions (Andrady, 2011).

The main chemical degradation pathway for aliphatic plastics (carbon-carbon backbone) such as PP and PE is byPOD (Gewert *et al.*, 2015), which requires oxygen and a light source. UV-B sunlight is primarily responsible for the initiation of POD in natural environments (Pospíšil and Nešpůrek, 1997). For POD to occur, polymers must contain unsaturated chromophoric groups which are capable of absorbing UV wavelengths of sunlight (Gijsman *et al.*, 1999). Polyolefins such as PE and PP theoretically do not contain these chromophoric groups in the polymer backbone. However, in practice trace, impurities develop in the polymers due to free radicals forming when the plastic is in a molten state while being manufactured, leading to the development of functional groups such as ketones and aldehydes, which can react with oxygen to form hydroperoxides and thus allow for POD to be initiated (Scott, 2002). POD of plastic can occur through a chain process initiated by free radicals that are produced after the carboxylic acid groups absorb UV light (Figure 1.2 Equation 1;Guillet (1978). Norrish Type I occur when aldehyde and ketone groups absorb UV (280-290 nm) (Wypych, 2008). Norrish

Type I reactions result in chain scission of the polymer and the formation of acyl and alkyl radicals (Figure 1.2 Equation 2). Norrish Type II reactions results in the formation of methyl ketones and alkenes (Figure 1.2 Equation 3).

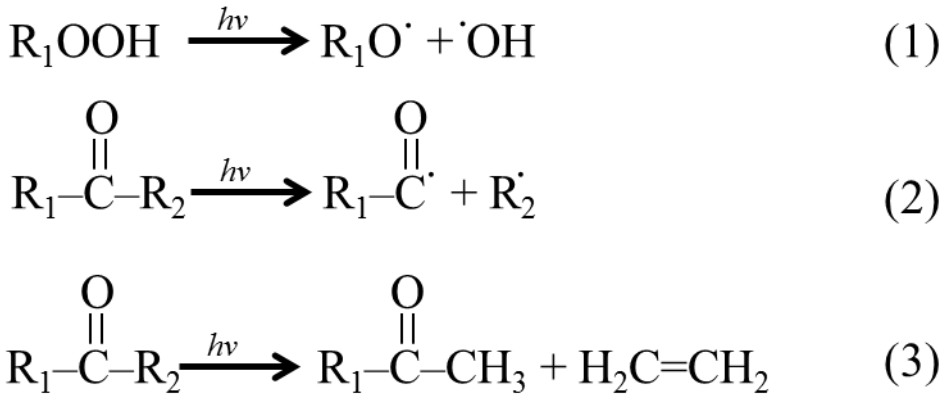


Figure 1.2 Common photo-oxidative degradation (POD) pathways of plastics. 1) Generalised photo-oxidation of acarboxylic acid group. 2) Norrish Type I reaction. 3) Norrish Type II reaction. Adapted from (Guillet, 1978)

The process of POD can be accelerated when plastics are present on sandy beaches, due to the high heat capacity of sand, which causes the plastics to heat (Andrady, 2011, Song *et al.*, 2017). However, the process of POD is still a slow process, and can be inhibited by anti-oxidants and stabilisers(Jakubowicz, 2003), and in aquatic environments due to the cooling effects of water (Pegram and Andrady, 1989).POD of plastic in water can be inhibited even further due to development of biofilms on the plastic surfaces, which are able to prevent light from reaching the plastic as well as possibly causing the plastic to become less buoyant and sink (Kooi *et al.*, 2017). As a result of random chain scission, end-chain scission, crosslinking, branching and/or the formation of oxygen-rich functional groups duringPOD, the hydrophobicity of the plastic surface decreases (Andrady, 2011). A positive feedback loop

of degradation is created, whereby the molecular weight of the polymer is reduced, the polymer becomes embrittled and fragments, which in turn, increases the surface area of the plastic and the rate of POD (Gewert *et al.*, 2015).The cracking fragmenting of the polymers may also increase the rate of which chemical additives (that are used to give plastics structural properties such as malleability) can leach out of the plastic matrix, causing the plastic to become brittle (Oehlmann *et al.*, 2009).

Heteroatom polymers such as PET and PLA do not have a carbon-carbon backbone (Corneillie and Smet, 2015, Gewert *et al.*, 2015). These may contain ester-bonds which can be susceptible to hydrolysis (Müller *et al.*, 2001). Additional hydrolysable covalent bonds include: ether, anhydride, amide, carbamide and ester amide bonds (Lucas *et al.*, 2008). Hydrolysis is affected by temperature, pH and the crystallinity and rate of diffusion of water molecules into amorphous regions of the polymer (Lucas *et al.*, 2008) and varies over time. Hydrolysis occurs more readily under acid or basic conditions (Allen *et al.*, 1991). Therefore, the hydrolysis of polymers may be autocatalytic when functional groups such as carboxylic acid or hydroxyl groups form (as well as through POD processes) which are able to catalyse hydrolysis of ester bonds(De Jong *et al.*, 2001, Gewert *et al.*, 2015).

1.5.2 Biological degradation pathways of plastic

Biological degradation of petroleum-based plastics has been reported to occur under controlled laboratory conditions (Table 1.3). Of the 75known microbial speciesthat have been identified in this literature review that have the potential to degrade plastics, 43 were bacteria and 32 were fungi. 67 of these organisms were isolated either from soil, compost, activated sludge or sediments, while only eightspecies were isolated directly from waste water or seawater namely: *Lysinibacillus fusiformis* (Mukherjee *et al.*, 2016),*Zalerion maritimum* (Paço *et al.*, 2017), *Pseudomonas fluorescens* (Howard and Blake, 1998), *Pseudomonas*

alcaligenes (Kim *et al.*, 2005),*Pseudomonas pachastrellae*(Sekiguchi *et al.*, 2011)*Alcanivorax venustensis* (Sekiguchi *et al.*, 2011)*Tenacibaculum soleae*(Sekiguchi *et al.*, 2011)and *Rhodospirillum rubrum* (Sznajder and Jendrossek, 2011). The bacterium *Ideonella sakaiensis* 201-F6 was isolated from marine sediment and in a consortium with a protozoan and a "yeast-like cell" and was able to degrade low crystalline (1.9%) PET films (Yoshida *et al.*, 2016).The predominance of the microorganisms isolated from soil environments when compared to aquatic environments may represent a sampling bias since more studies investigating the potential for microorganisms to degrade plastics from soil samples when compared to aquatic samples have been published (32 and 5 studies identified through this literature review, respectively). The enzymes identified to be responsible were PETase and MHETase(Yoshida *et al.*, 2016), which exhibit typical hydrolase structures and attack the ester-linkages in the PET (Austin *et al.*, 2018). The Yoshida *et al.* (2016) study has been criticised for using a low crystalline PET film (Yang *et al.*, 2016), because the crystallinity of a polymer is a major factor in its biodegradability, with lower crystalline polymers being more readily degradable (Tokiwa *et al.*, 2009). During the biodegradation process of heteroatom plastics such as PET and PLA, hydrolysis of the ester bonds occurs (Tokiwa and Calabia, 2006).

Table 1.3Microorganisms associated with plastic degradation, grouped by polymer chemical structure, (carbon-carbon backbone plastics or heteroatom plastics) and microbial group.

Microbial group	Organism	Polymer type*	Organism Source or Environment	Reference
Carbon-carbon backbone plastics				
Bacteria	*Acinetobacter baumannii*	PE	Soil	(Pramila *et al.*, 2012)
Bacteria	*Achromobacter xylosoxidans*	PE	Soil	(Kowalczyk *et al.*, 2016)
Bacteria	*Bacillus sp.* YP1	PE	Waxworm (*Plodia interpunctella*) stomach	(Yang *et al.*, 2014)
Bacteria	*Bacillus amyloliquefaciens* BSM-1	PE	Soil from landfill	(Das and Kumar, 2015)
Bacteria	*Bacillus amyloliquefaciens* BSM-2	PE	Soil from landfill	(Das and Kumar, 2015)
Bacteria	*Bacillus circulans*	PE	Soil	(Watanabe *et al.*, 2009)
Bacteria	*Bacillus brevies*	PE	Soil	(Watanabe *et al.*, 2009)
Bacteria	*Bacillus sphaericus*	PE	Soil	(Watanabe *et al.*, 2009)
Bacteria	*Brevibacillus borstelensis*	PE	Soil	(Hadad *et al.*, 2005)
Bacteria	*Brevibacillus parabrevis*	PE	Soil	(Pramila *et al.*, 2012)
Bacteria	*Chelatococcus daeguensis*	PE	Compost	(Jeon and Kim, 2013)
Bacteria	*Enterobacter asburiae* YTI	PE	Waxworm (*Plodia interpunctella*) stomach	(Yang *et al.*, 2014)
Bacteria	*Lysinibacillus fusiformis*	PE	Waste water	(Mukherjee *et al.*, 2016)
Bacteria	*Pseudomonas citronellolis* EMBSO27	PE	Soil	(Bhatia *et al.*, 2014)
Bacteria	*Rhodococcus rubber*	PE	Soil	(Gilan *et al.*, 2004)
Fungus	*Aspergillusflavus* VRKPT2	PE	Soil	(Sangeetha Devi *et al.*, 2015)
Fungus	*Aspergillus nidulans*	PE	Soil	(Usha *et al.*, 2011)
Fungus	*Aspergillustubingensis* VRKPT1	PE	Soil	(Sangeetha Devi *et al.*, 2015)
Fungus	*Curvularia lunata*	PE	Soil from landfill	(Raut *et al.*, 2015)
Fungus	*Fusariurn redolens*	PE	Soil	(Albertsson, 1978)
Fungus	*Penicillium simplicissimum* YK	PE	Soil	(Yamada-Onodera *et al.*, 2001)

Fungus	*Pleurotus ostreatus* (Oyster mushroom)	Oxo-biodegradable PE	Trees	(Da Luz *et al.*, 2014)
Fungus	*Rhizopus oryzae* NS5	PE	Soil	(Awasthi *et al.*, 2017)
Fungus	*Trichoderma harzianum*	PE	Soil from landfill	(Sowmya *et al.*, 2014)
Fungus	*Zalerion maritimum*	PE	Seawater	(Paço *et al.*, 2017)
Fungus	*Engyodontium album*	PP	Soil	(Jeyakumar *et al.*, 2013)
Fungus	*Phanerochaete chrysosporium*	PP	Soil	(Jeyakumar *et al.*, 2013)
Fungus	*Lasiodiplodia theobromae*	PP	Roots of *Psychotria flavida*	(Sheik *et al.*, 2015)
Bacteria	*Rhodococcus opacus* 1CP	PS	Soil	(Oelschlägel *et al.*, 2012)
Bacteria	*Pseudomonas putida*E-93486	PS	Activated sludge	(Gąszczak *et al.*, 2012)
Bacteria	*Pseudomonas putida* NBUS12	PS	Activated sludge	(Tan *et al.*, 2015)
Bacteria	*Ochrobactrum* TD	PVC	Soil from landfill	(Danko *et al.*, 2004)
Bacteria	*Pseudomonas putida* AJ	PVC	Soil from landfill	(Danko *et al.*, 2004)
Fungus	*Aureobasidium pullulans*	PVC	Outside atmosphere	(Webb *et al.*, 2000)
		Heteroatom plastics		
Bacteria	*Ideonella sakaiensis*	PET	Marine sediment	(Yoshida *et al.*, 2016)
Bacteria	*Thermobifida alba* Est119	PET-copolymer Apexa®	Compost	(Hu *et al.*, 2010)
Bacteria	*Comamonas acidovorans* TB-35	PUR	Soil	(Akutsu *et al.*, 1998)
Bacteria	*Pseudomonasfluorescens*	PUR	Water	(Howard and Blake, 1998)
Fungus	*Aureobasidium pullulans*	PUR	Soil	(Crabbe *et al.*, 1994)
Fungus	*Cladosporium sp.*	PUR	Soil	(Crabbe *et al.*, 1994)
Fungus	*Curvularia senegalensis*	PUR	Soil	(Crabbe *et al.*, 1994)
Fungus	*Fusarium solani*	PUR	Soil	(Crabbe *et al.*, 1994)
Fungus	*Pestalotiopsis microspora*	PUR	Plants	(Russell *et al.*, 2011)
Fungus	*Aspergillus oryzae.*	PBS	Soil	(Maeda *et al.*, 2005)
Bacteria	*Bacillus sp.*	PHA	Seawater	(Volova *et al.*, 2011)
Bacteria	*Enterobacter cloacae*	PHA	Seawater	(Volova *et al.*, 2011)
Bacteria	*Gracilibacillus sp.*	PHA	Seawater	(Volova *et al.*, 2011)

Bacteria	*Pseudomonas lemoignei*	PHB	Soil	(Delafield *et al.*, 1965)
Bacteria	*Pseudomonas alcaligenes*	PHB	Seawater	(Kim *et al.*, 2005)
Bacteria	*Rhodospirillum rubrum*	PHB	Lake surface water	(Sznajder and Jendrossek, 2011)
Bacteria	*Streptomyces ascomycinicus*	PHB	Soil	(García-Hidalgo *et al.*, 2013)
Fungus	*Pseudozyma antarctica* JCM	PHB	Soil	(Shinozaki *et al.*, 2013)
Bacteria	*Pseudomonas pachastrellae*	PCL	Seawater	(Sekiguchi *et al.*, 2011)
Bacteria	*Alcanivorax venustensis*	PCL	Seawater	(Sekiguchi *et al.*, 2011)
Bacteria	*Tenacibaculumsoleae*	PCL	Seawater	(Sekiguchi *et al.*, 2011)
Fungus	*Aspergillus flavus*	PCL	Soil	(Benedict *et al.*, 1983)
Fungus	*Aspergillus fumigatus*	PCL	Soil	(Benedict *et al.*, 1983)
Fungus	*Aspergillus niger*	PCL	Soil	(Benedict *et al.*, 1983)
Fungus	*Chaetomium globosum*	PCL	Soil	(Benedict *et al.*, 1983)
Fungus	*Fusarium sp.*	PCL	Soil	(Benedict *et al.*, 1983)
Fungus	*Pencillium funiculosum*	PCL	Soil	(Benedict *et al.*, 1983)
Fungus	*Penicillium oxalicum* DSYD05-1	PCL	Soil	(Li *et al.*, 2012)
Fungus	*Pseudozyma japonica* Y709	PCL	Plants	(Abdel-Motaal *et al.*, 2014)
Bacteria	*Actinomadura keratinilytica*	PLA	Soil	(Sukkhum *et al.*, 2009)
Bacteria	*Amycolatopsis orientalis*	PLA	Soil	(Pranamuda and Tokiwa, 1999)
Bacteria	*Amycolatopsis tolypophorus*	PLA	Soil	(Jarerat *et al.*, 2002)
Bacteria	*Amycolatopsis mediterranei*	PLA	Soil	(Jarerat *et al.*, 2002)
Bacteria	*Amycolatopsis azurea*	PLA	Soil	(Jarerat *et al.*, 2002)
Bacteria	*Bacillus brevis*	PLA	Soil	(Tomita *et al.*, 1999)
Bacteria	*Bacillus licjeniformis*	PLA	Soil	(Sukkhum *et al.*, 2009)
Bacteria	*Bacillus stearothermophilus*	PLA	Soil	(Tomita *et al.*, 2003)
Bacteria	*Bordetellapetrii*	PLA	Soil	(Kim and Park, 2010)
Bacteria	*Laceyella sacchari*	PLA	Soil	(Sukkhum *et al.*, 2009)
Bacteria	*Nonomuraea fastidiosa*	PLA	Soil	(Sukkhum *et al.*, 2009)
Bacteria	*Nonomuraea terrinata*	PLA	Soil	(Sukkhum *et al.*, 2009)

Bacteria	*Micromonspora viridifaciens*	PLA	Soil	(Sukkhum *et al.*, 2009)
Bacteria	*Saccharothrix waywayandensis*	PLA	Soil	(Jarerat and Tokiwa, 2003)
Bacteria	*Thermoactinomyces vulgaris*	PLA	Soil	(Sukkhum *et al.*, 2009)
Fungus	*Rhizopus delemer*	PLA	Soil	(Fukuzaki *et al.*, 1989)
Fungus	*Penicillium roquefort*	PLA	Soil	(Pranamuda *et al.*, 1997)
Fungus	*Fusarium moniliforme*	PLA	Soil	(Torres *et al.*, 1996)
Fungus	*Pseudozyma antarctica* JCM	PLA	Soil	(Shinozaki *et al.*, 2013)
Fungus	*Tritirachium album*	PLA	Soil	(Jarerat and Tokiwa, 2001)
Fungus	*Trichoderma viride*	PLA	Soil	(Lipsa *et al.*, 2016)
Fungus	*Pseudozyma antarctica* JCM	PBSA	Soil	(Shinozaki *et al.*, 2013)
Fungus	*Rhizopus oryzae*	"Mater-Bi carrier bags"	Soil	(Accinelli *et al.*, 2012)

* PE = Polyethylene, PP = Polypropylene, PS= Polystyrene, PVC = Polyvinyl chloride, PET = Poly(ethylene terephthalate), PUR = Polyurethane, PBS = Polybutylene succinate,PHA = Polyhydroxyalkanoate,PHB = Polyhydroxybutyrate, PCL = Polycaprolactone,PLA = Polylactic acid, PBSA = Polybutylenes succinate-co-adipate.

1.6 Ecological impacts of plastic

There has been extensive and growing research over the last two decades into the impact, fate and sources of plastics in the marine environment (Laist, 1997). The two main ways by which plastic debris physically affect marine life are by entanglement, whereby the organism becomes trapped and by ingestion either passively by filter feeders or actively by organisms such as birds(Gregory, 2009). At least 395 different species have been documented that have either ingested plastic or been entangled by plastic (Gall and Thompson, 2015). As this thesis is focused on smaller plastics fragments, only the impacts of plastic ingestion will be discussed further.

Ingestion of either macroplastic or microplastic debris is now a common occurrence by marine and freshwater organisms and is well documented(Browne *et al.*, 2008, Fossi *et al.*, 2012, Cole *et al.*, 2013, Setälä *et al.*, 2014, Nelms *et al.*, 2018). A high prevalence of plastic has been found in the low-trophic level organism Japanese anchovy (*Engraulis japonicus*) (>75%), in the highly urban and industrialised Tokyo Bay, Japan (Tanaka and Takada, 2016).In the African Great lake, Lake Victoria, 20% of the commercially important Nile perch (*Lates niloticus*), a high-trophic-level fish, and Nile tilapia (*Oreochromis niloticus*) were found to have consumed microplastics from a sample of 40 fish (Biginagwa *et al.*, 2016).While in the oceanic gyres, mesopelagic fish may be a major sink for microplastics as it is estimated that they consume between 12,000 and 24,000 tons year^{-1}of plastic(Davison and Asch, 2011, Choy and Drazen, 2013).

The ingestion of plastic has been associated with a broad range of negative impacts. The ingestion of plastic can cause physical harm to the organism once ingested by causing blockages, scarring and ulcerations to the intestinal tract(Ryan, 1987).Laboratory experiments have shown that after the zooplankton *Centropages typicus* had consumedpolystyrene

microbeads they ingested fewer algae putting them at an energy loss (Cole *et al.*, 2013).Based on a meta-analysis of 186 seabird species, by 2050, 99% of all seabird species are expected to consume plastic (Wilcox *et al.*, 2015). Intergenerational transfer of plasticfrom the adults to the chicks has been observed inbirds such asShort-tailed Shearwaters (*Ardenna tenuirostris*) (Carey, 2011) and Black-footed Albatrosses (*Phoebastria nigripes*)(Rapp *et al.*, 2017). Reduced body mass and head bill length has been correlated with plastic ingestion ofFlesh-footed Shearwater (*Puffinus carneipes*) fledglings (Lavers *et al.*, 2014). It is believed that seabirds confuse plastic for similar looking food such as fish eggs and jellyfish(Bond *et al.*, 2013).Olfactory sensors in seabirds may also be triggered due to the chemicals emitted by the biofouling algae on plastic surfaces(Savoca *et al.*, 2016). However, the experimental methods and interpretations of this study have been brought into disrepute, due to misclassification of species, and the experimental observations employed(Dell'Ariccia *et al.*, 2017).

Chemical pollutants may transfer from ingested plastic to the organisms, leading to the ingestion of plastics being likened to a 'chemical cocktail' (Rochman, 2013), with plastics reported to adsorb over 230 chemicals from the ocean (Rani *et al.*, 2015). These chemicals include additives(Koelmans *et al.*, 2014), metals (Ashton *et al.*, 2010, Holmes *et al.*, 2012)and persistent organic pollutants (POPs) (Rochman *et al.*, 2013b)that have been found tobe adsorbed onto the polymer surface. There is still ongoing debate over how large an exposure pathway this represents when compared to other POP transport mechanisms such as uptake from water or through the gills (Gouin *et al.*, 2011, Engler, 2012).

1.7 Chemical pollutants associated with plastic debris

1.7.1 Persistent organic pollutants (POPs) associated with plastic debris

Persistent organic pollutants (POPs) are a class of organic chemicals that are toxic, can bioaccumulate, are highly stable in the environmentand are hydrophobic organic chemicals (HOCs)(Wania and Mackay, 1996, Bakir *et al.*, 2016). A subset of these chemicals are recognised and regulated under the Stockholm Convention(United Nations Environment Programme, 2009, United Nations Environment Programme, 2017). Under the convention there were originally 12 chemicals recognised.However, since the initial ratification, 16 more chemicals have been added (United Nations Environment Programme, 2017). The original 12 chemicals covered by the Stockholm Convention included; pesticides including the infamous dichlorodiphenyltrichloroethane (DDT) which had been strongly associated with population decreases in birds(Keith, 1966); industrial chemicals hexachlorobenzene and polychlorinated biphenyls (PCBs) which were used in electrical transformers; and chemicals produced as accidental by-products included both hexachlorobenzene and PCBs as wells as polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDF). PCDD/PCDFs were produced as a by-product of the defoliant Agent Orange, that was used extensively by the U.S.A during the Vietnam War (Mukerjee, 1998) and is a teratogen(Brouwer *et al.*, 1995). The additional POPs covered by the extended Stockholm Convention list include polybrominated diphenyl ethers (PBDEs) which have been used as flame retardants in furniture, plastics and electrical and electronic goods(Rahman *et al.*, 2001).

POPs with high octanolwater partition coefficients (K_{ow}) are likely to adsorb onto plastic surfaces due to their hydrophobic propertiesin marine environments(Teuten *et al.*, 2007). POPs have been detected on plastics recovered from beaches near highly urban environments (Zhang et al., 2015b) as well as in the remote oceanic gyres(Hirai *et al.*, 2011, Rios Mendoza and Jones, 2015, Chen *et al.*, 2018). The primary compounds of interest identified in this

literature reviewfrom most of the studies that have sought to quantify POPs on plastic from marine environments have beenPCBs, and polycyclic aromatic hydrocarbons (PAHs). Lesser emphasis has been placed on brominated flame retardants (BFRs) such as PBDEs or on a class of novel brominated flame retardants (NBFRs). NBFRs are a broad group of BFRs that have been defined as being new to market (Covaci *et al.*, 2011), other suitable terms include "non-PBDE BFRs" or "current-use BFRs" (Covaci *et al.*, 2011).Upon an extensive review of the available literature, the concentrations of PCBs, PAHs and PBDEs from plastic sourced from marine environments are presented inTable 1.4. The concentrations ofPCBs and PBDEs on plastic debris in marine environmentstypically ranged between 1- 10,000 ng g^{-1}, whilst PAHs typically ranged between 1- 120,000ng g^{-1}. Plastics collected from theHuanghai Sea, China, had the highest reported concentrations of PAHs (120,00 ng g^{-1})(Mai *et al.*, 2018).

Table 1.4 Concentrations of brominated flame retardants, PCBs and PAHs adsorbed to plastics sourced directly from the marine environment.

Analytes	**Plastic Type**	**Range (ng g^{-1})**	**Source**	**Location**	**Reference**
PBDE (∑20 congeners)	PP and PE	0.02- 9,900	Trawl	Central Pacific Gyre	(Hirai *et al.*, 2011)
PBDE(∑15 congeners)	Fragment	0.6-188	Trawl	North Pacific Gyre	(Chen *et al.*, 2018)
HBCD (∑4congeners)	Fragment	0.01-740	Trawl	North Pacific Gyre	(Chen *et al.*, 2018)
PCB (∑15 congeners)	Pellet	0.02-16	Beached litter	Portugal	(Frias *et al.*, 2010)
PCB (∑7 congeners)	Pellet	<LOD- 210	Beached litter	Hawaii	(Heskett *et al.*, 2012)
PCB (∑13 congeners)	Pellet	61-113	Beached litter	South Africa	(Ryan *et al.*, 2012)
PCB (∑13 congeners)	Pellet	0.1-9.9	Beached litter	India	(Jayasiri *et al.*, 2015b)
PCB (∑23 congeners)	PP	4-117	Beached litter	Japan	(Mato *et al.*, 2001)
PCB (∑13 congeners)	PE	32-605	Beached litter	USA	(Ogata *et al.*, 2009)
PCB (∑13 congeners)	PE	169-453	Beached litter	Japan	(Ogata *et al.*, 2009)
PCB (∑6 congeners)	Fragment	<LOD- 47	Beached litter	USA	(Van *et al.*, 2012)
PCB (∑20 congeners)	Pellet	2-22	Beached litter	Portugal	(Antunes *et al.*, 2013)
PCB (∑27 congeners)	PE	25-34	12 month marine exposure experiment	USA	(Rochman *et al.*, 2013a)
PCB (∑27 congeners)	PP	8-25	12 month marine exposure experiment	USA	(Rochman *et al.*, 2013a)
PCB (∑7 congeners)	Pellet	31-236	Beached litter	Belgium	(Gauquie *et al.*, 2015)
PCB (∑7 congeners)	Pellet	<LOD-210	Beached litter	India	(Jayasiri *et al.*, 2015a)
PCB (∑34 congeners)	Fragment	1-223	Trawl	North Pacific Gyre	(Rios Mendoza and Jones, 2015)
PCB (∑20 congeners)	Pellet	34-323	Beached litter	China	(Zhang *et al.*, 2015b)
PCB (∑13 congeners)	Pellet	107-294	Beached litter	Australia	(Yeo *et al.*, 2015)

PCB (∑13 congeners)	Pellet	4-24	Beached litter	Vietnam	(Le *et al.*, 2016)
PCB (∑51 congeners)	Pellet	3-7,554	Beached litter	Brazil	(Taniguchi *et al.*, 2016)
PCB (∑28 congeners)	Fragment	0.7-455	Trawl	North Pacific Gyre	(Chen *et al.*, 2018)
PAH (∑15 compounds)	Pellet	0.2-319	Beached litter	Portugal	(Frias *et al.*, 2010)
PAH (∑16 compounds)	Fragment	30-1,900	Beached litter	USA	(Van *et al.*, 2012)
PAH (∑17 compounds)	Pellet	53- 44,800	Beached litter	Portugal	(Antunes *et al.*, 2013)
PAH (∑12 compounds)	Pellet	130-27,735	Beached litter	Brazil	(Fisner *et al.*, 2013)
PAH (∑15 compounds)	PE	80- 1,000	12 month marine exposure experiment	USA	(Rochman *et al.*, 2013a)
PAH (∑15 compounds)	PP	5-100	12 month marine exposure experiment	USA	(Rochman *et al.*, 2013a)
PAH (∑16 compounds)	Pellet	1,076-3,007	Beached litter	Belgium	(Gauquie *et al.*, 2015)
PAH (16 compounds)	Fragment	1-846	Trawl	North Pacific Gyre	(Rios Mendoza and Jones, 2015)
PAH (∑16 compounds)	Pellet	136-2,384	Beached litter	China	(Zhang *et al.*, 2015b)
PAH (∑16 compounds)	Pellet	192- 13,708	Beached litter	Brazil	(Taniguchi *et al.*, 2016)
PAH (∑15 compounds)	Fragment	1- 848	Trawl	North Pacific Gyre	(Chen *et al.*, 2018)
PAH (∑16 compounds)	Pellet	<LOD-1,592	Beached litter	Greece	(Karkanorachaki *et al.*, 2018)
PAH (∑16 compounds)	Pellet	3400-120,000	Trawl	China	(Mai *et al.*, 2018)

PBDE= polybrominated diphenyl ethers,HBCD =Hexabromocyclododecane,PCB = polychlorinated biphenyls, PAH =polycyclic aromatic hydrocarbons, LOD = Limit of detection.

1.7.2 Plastic additives of concern

Modern synthetic plastics containchemical additives, which are added to alter the polymer properties.These include, altering heat resistance, strength, malleability, antioxidant properties and reducing static electricity effects (Thompson *et al.*, 2009). In a chemical qualitative analysis of 43 plastic samples (25 new plastics, 18 beached debris) over 50 different plastic additives were identified (Rani *et al.*, 2015). Some of these chemicals have been associated with adverse effects in humans and other organisms and are outlined inTable 1.5. Plastic additives such as Bisphenol-A (BPA), PBDEs and NBFRs are not chemically bound to the plastic matrix; and may leach from the plastics into the environment, or transfer to the user of the plastic item (Oehlmann *et al.*, 2009). This is an environmental concern because of the ease of leaching of these additives from plastics and their potential adverse effects on wildlife, such as DNA damage in Fathead minnows (*Pimephales promelas*)(Bearr *et al.*, 2010), or the accumulation of additives in abdominal adipose of sea birds (Tanaka *et al.*, 2015). The ingestion of plastics containing PBDEs by fish has led to an increase in hepatic stress (Rochman *et al.*, 2013b), and may cause other endocrine effects such as limiting thyroid function (Yogui and Sericano, 2009). The leaching of additives from plastics is influenced by a range of factors including from the hydrophobicity/ hydrophilicity of the plasticizers, the glass interface of the plastic, the size of the additive and the pore size of the polymer in which the additive sits (Teuten *et al.*, 2009).

BPA is a plastic additive that has received a lot of media attention, because it has been shown to act as an endocrine disrupting chemical (EDC), in particular in rodent foetuses (Vandenberg *et al.*, 2009). Exposure to BPA at critical foetal developmental stages can lead to greater susceptibility to diseases in adulthood (Vandenberg *et al.*, 2009). The use of BPA bottles was banned in Australia in 2010, after they had been phased out in the U.S.A (Food Standards Australia New Zealand (FSANZ), 2016).

Table 1.5Plastic additives their uses and potential adverse effects

Plastic additive	**Abbreviation**	**Potential use**	**Association with potential adverse effect**	**Citation**
Bisphenol-A	BPA	Polycarbonate plastics or PVC stabiliser	Oestrogen mimicker	(Teuten *et al.*, 2009)
Phthalates	-	PVC stabiliser	Lower testosterone and anti-androgenic	(Meeker *et al.*, 2009)
Short-chain chlorinated paraffins	SCCP	Flame retardant	Toxicity demonstrated in human HepG2 cells	(Geng *et al.*, 2015)
Bis(tributyltin)oxide	TBTO	Anti-microbial paints	Toxic to aquatic organisms	(Ward *et al.*, 1981)
Tricloscan	-	Anti-microbial	Breast cancer and prostate cancer	(Gee *et al.*, 2008)
Polybrominated diphenyl ethers	PBDEs	Brominated flame retardants	Limits thyroid function	(Linares *et al.*, 2015)

PBDEs and NBFRs are added to plastic products to act as flame retardants, they are ubiquitous in household furniture and appliances such as mattresses and circuit boards. They tend to have low water solubility, high lipophilicity and are semi-volatile (Andrady, 2011). PBDEs are solely anthropogenic in origin and are highly persistent in the environment, being found in remote regions such as Arctic water samples and in the tissue of apex predators such as Polar bears (*Ursus maritimus*) (de Wit *et al.*, 2006). PBDEs are leached from plastics by stomach oil in seabirds belonging to the order of Procellariiformes(Tanaka *et al.*, 2015). PBDEs are also widely found in human populations, a study of breast milk from 108 women in China, ∑8 PBDE congeners ranged between 0.22 to 135.41 ng g^{-1}lipid weight (lw), whilst the brominated flame retardant tetrabromobisphenol A (TBBPA) ranged from <limit of detection (LOD) to 12.46 ng g^{-1}lw, and the ∑3 hexabromocyclododecane (HBCD) congeners ranged from <LOD to 78.28 ng g^{-1} lw(Shi *et al.*, 2013). Due of the extent of plastic pollution, there is concern that the amount of plastic additives released into the aquatic and marine

environments will increase over time. This may lead to increased incidence of adverse effects associated with these plastic additives.

The magnitude of the risk of POP exposure to organisms from plastics when compared to other exposure pathways such as via direct ingestion of sediment or direct transfer across the gills is currently in dispute (Zarfl and Matthies, 2010, Gouin *et al.*, 2011, Browne *et al.*, 2013, Koelmans *et al.*, 2016). However, these previous studies have been criticised for relying on equilibrium partitioning, and have not taken into account factors such asgut surfactants, pH or temperature or variable plastic residence times in organisms(Bakir *et al.*, 2016).For instance, a leaching experiment of PBDEs (congener BDE-209) from PE plastics using seabird stomach oil and fish oil found that the oils leached between 20 and 50 times the amount of BDE-209 when compared to a water solution, respectively (Tanaka *et al.*, 2015). Conversely, a correlation between increased plastic consumption and increased POPbody burden has often not been established (Herzke *et al.*, 2015, Besseling *et al.*, 2017, Clukey *et al.*, 2018). It is assumed that POPs within the organism accumulate over the lifespan of the organism(Tuerk Karen *et al.*, 2009).However,egestion rates of plastic remain largely unknown (Wright *et al.*, 2013). In laboratory experiments, juvenile planktivorous fish, Palm ruff, (*Seriolella violacea*) were found to egest microplastics within an average of seven days (Ory *et al.*, 2018). The residence of plastic in tadpoles (*Xenopus tropicalis*) has been found to be as low as six hours (Hu *et al.*, 2016) and as low as 12 hours in mysid shrimp (*Neomysis integer*) (Setälä *et al.*, 2014). This causes uncertainty when attempting to correlate the amount of plastic in stomach contents and the proportion of POPs derived from these plastics. It has been suggested that the ecological risks of POP transfer from plastic to an organism are likely to be both plastic- and chemical- specific (Bakir *et al.*, 2016). It should also be noted that there are now calls for plastics to be categorised as POPs themselves since plastic pollutants meet the Stockholm

Conventionrequirements of being persistent, to bioaccumulate, to undergo long-range transport and to cause adverse effects to organisms(Lohmann, 2017).

1.8 Microbial biofilms on plastic

1.8.1 The role of microbial biofilms in key environmental functions

Marine microbial biofilms fill key environmental functions, they are critical for the cycling of nutrients (including nitrogen, phosphorus and sulphur), are sites of key primary production, support larger food-websand secrete extracellular polymeric substances (EPS) onto surfaces which is followed by the attachment of the larvae of higher fouling organisms such as barnacles and mussels(Patil and Anil, 2005).

Biofilms are defined as a matrix-enclosed microbial populations(bacteria, archaea and fungi) adherent to each other and/or to surfaces or interfaces (Costerton *et al.*, 1995); biofouling communities by comparison are dominated by larger organisms such as barnacles and mussels (Callow and Callow, 2002). Biofilms on ships have the potential to harbour pathogens and invasive species and transfer them into a local environment as ships move between ports (Drake *et al.*, 2007).A high profile example in Australia is the introduction of the North Pacific sea star (*Asterias amurensis*) (Byrne *et al.*, 1997), which led to the decline in large bivalve numbers in Tasmania (Ross *et al.*, 2002).The spread of marine invasive species is recognised as one of the largest ecological and economical threats to the world's oceans (International Marine Organisation (IMO), 2017). The risks are believed to be so great that the International Marine Organisation (IMO) introduced ANNEX 26, which aims to control and manage invasive species transfer from ships (International Marine Organisation (IMO), 2011).

1.8.2 Microbial biofilms associated with plastic debris

In 1972, microorganisms were first observed on floating pelagic plastic debris (Carpenter *et al.*, 1972). Since then, the development of culture-independent biomolecular techniques have allowed researchers to look more deeply into the microbial community composition of plastic

biofilms (Zettler *et al.*, 2013, Harrison *et al.*, 2014, Oberbeckmann *et al.*, 2014); these and other studies are reviewed in depth in Chapter 5. Plastic microbial biofilms have been found to be structurally and functionally distinct from the free-living communities in surrounding aquatic environments (McCormick *et al.*, 2016, Arias-Andres *et al.*, 2018, Dussud *et al.*, 2018), with diverse communities varying with spatial(Hoellein *et al.*, 2014), temporal(Pollet *et al.*, 2018)and seasonal (Oberbeckmann *et al.*, 2014)factors.

Aquatic plastic debris have also been found to be highly productive surfaces and may facilitate the transport of pathogens(Bryant *et al.*, 2016). Biofilm communities on 5 mm pieces of plastic have been recorded to have chlorophyll-*a* concentrations rangingsimilar to those in30-700 mL of seawater (Bryant *et al.*, 2016).In a similar manner to transport via shipping, it is thought that plastic debris may act as a raft and aid in the transportation of pathogenic and invasiveprokaryotic and eukaryotic species through aquatic environments, although evidence for this is currently limited (Derraik, 2002, Gregory, 2009, Viršek *et al.*, 2017).

Under laboratory conditions it has been demonstrated that there is the potential forsome microorganisms within biofilms to facilitate the degradation of some plastics (see Section1.5.2). Bacteria have also been visualised inhabiting pits in the surface of plastic fragments recovered from the Sargasso Sea (Zettler *et al.*, 2013) and from coastal waters from around Australia (Reisser *et al.*, 2014)using scanning electron microscopy (SEM).Zettler *et al.* (2013)suggested that the bacteria inhabiting the pits in the plastic fragments could be evidence that the bacteria were degrading the plastic, and that further testing was required.

1.9 Research Aims and Objectives

With the release of plastic debris into aquatic environments expected to continue, the amount of plastic debris in oceans is expected to also continue to increase(Jambeck *et al.*, 2015). Simultaneously an increase in the use of bioplastics is occurring(European Bioplastics, 2017).Given the known impacts of petroleum-based plastics on aquatic animals(Gregory, 2009), it is also important to assess the potential fates and impacts of bioplastics.This study aimed to advance current knowledge of the fate of pollutant plastics within aquatic ecosystems by investigating changes in the physical and chemical structure of a petroleum-based plastic and a bioplastic and further investigating the adsorption of POPs onto polymer surfaces and compositional changes in plastic microbial biofilm communities, along an estuarine environment over time. To address this overarching aim, a 12-month long exposure experiment was established at five sites along a freshwater-marine continuum of the Yarra River into Port Phillip Bay, Melbourne, Australia; in which coupons of polypropylene (PP), polylactic acid (PLA) and glass (as a control substrate) were suspended in surface waters and sampled over time.

The three specific objectives of this thesis were to:

1. Compare variation in the structural properties of PP and PLA, via analysis of surface hydrophobicity, tensile strength, crystallinity and chemical structure (Chapter 3)
2. Determine the potential for brominated flame retardants (PBDEs and NBFRs) to adsorb and accumulate on PP and PLA with comparison to glass substrates (Chapter 4)
3. Compare spatial-, temporal- and substrate-specific (PP, PLA and glass) variation in the structure and composition of microbial (prokaryotic and eukaryotic) biofilm communities as well as water communities (Chapter 5)

Chapter 2: Experimental Design and sampling scheme

2.1 Experimental summary

To address the three research areas of this PhD (see Section1.9), a 12-month long exposure experiment was established at five sites along a freshwater-marine continuum of the Yarra River into Port Phillip Bay, Melbourne, Australia. Plastic substrates (polypropylene (PP) and polylactic acid(PLA)) and glass substrates (as a non-plastic control substrate) were suspended in surface waters, and periodically sacrificially sampled. Samples were then processed according to their respective analysis streams of physical, chemical or biological characterisation. The experimental setup is described below. Detailed methods for each analytical stream are outlined in each of the respective Results chapters (Chapters 3, 4 and 5).

2.2 Site descriptions

Site 1 to Site 4 were located in highly urbanised and industrialised reaches of the Yarra River; Site 5 was located in a harbour near the mouth of Yarra River where it meets Hobsons Bay (Figure 2.1). Briefly, Site 1 (Dights Falls) (37°47'45.8"S 145°00'07.3"E) was located approximately 150 m upstream of the Dights Falls weir which prevents the intrusion of saline tidal water into upstream waters. At Site 1, sample frames were secured to iron stakes located 1.5 m from the river bank. At Sites 2 (MacRobertson Bridge) (37°49'50.7"S 145°00'42.1"E) and Site 3 (Federation Square) (37°49'07.6"S 144°58'15.7"E) sample frames were secured to the downstream end of litter trap barges, operated by Parks Victoria. At Site 4 (Westgate Bridge) (37°49'45.3"S 144°54'01.4"E) sample frames were attached to a jetty, operated by Parks Victoria. Sample frames located at Site 5 (Williamstown) (37°47'45.8"S 145°00'07.3"E) were secured underneath a pier, operated by SeaWorks (Figure 2.2).

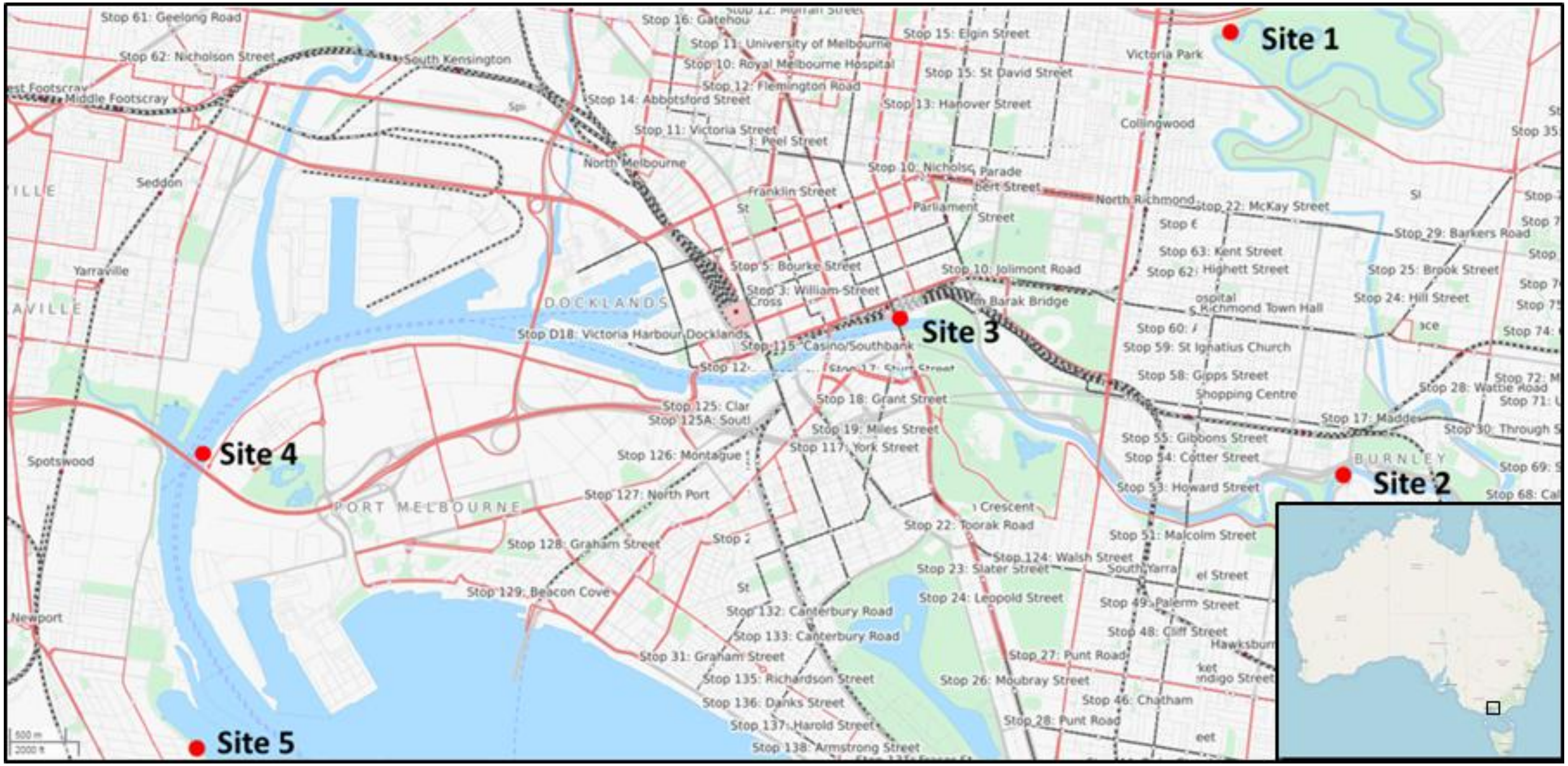


Figure 2.1 Mapof sample sites along the Yarra River. Site 1) Dights Falls, Site 2) MacRobertson Bridge, Site 3) Federation Square, Site 4) Westgate Bridge, Site 5) Williamstown. Insert is of the larger Australian map indicating the location of the sample sites. Map is orientated with north being at the top of the image. Source: OpenStreetMap.

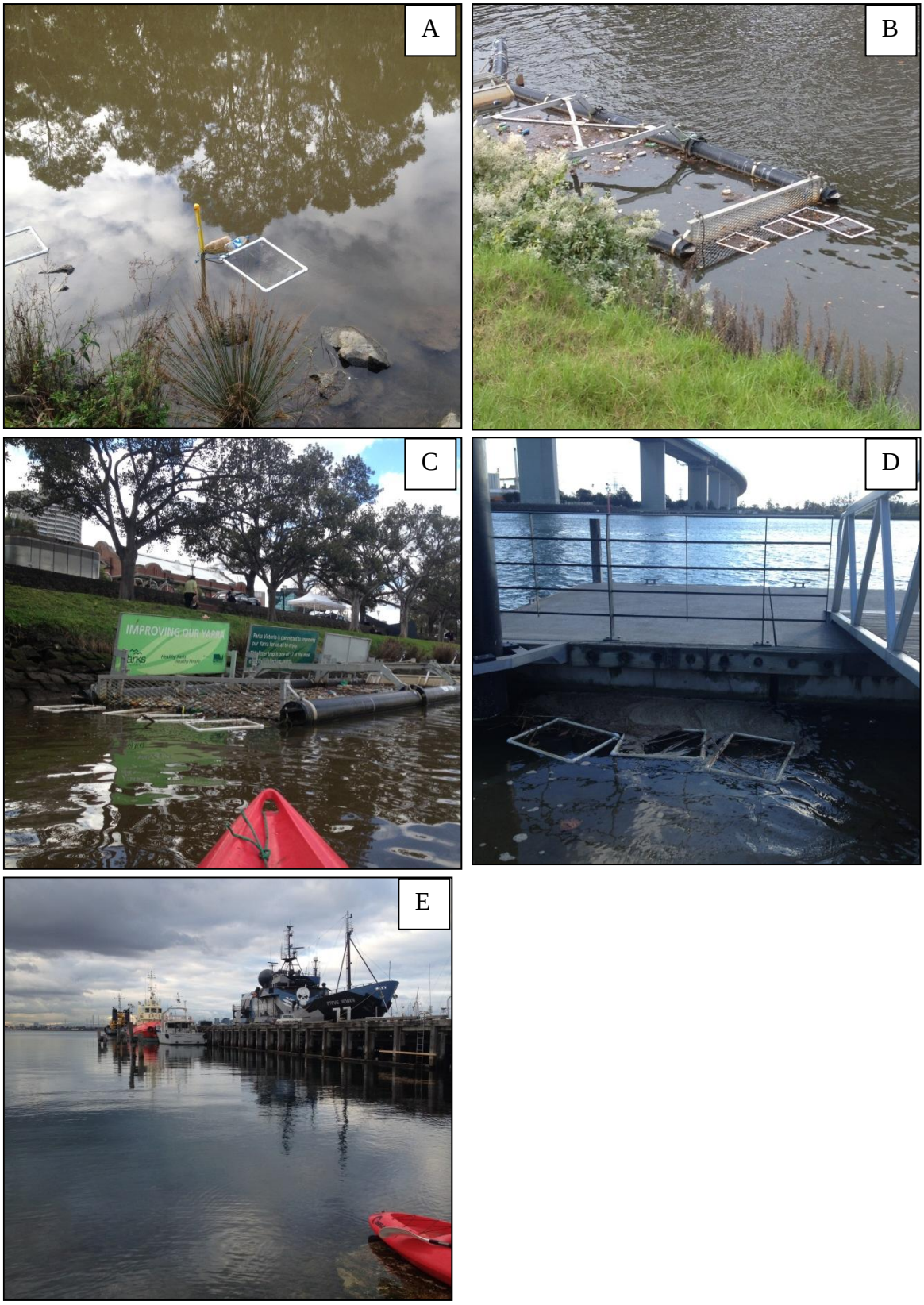


Figure 2.2 Images taken of sample sites. A) Dights Falls B) MacRobertson Bridge C) Federation Square D) Westgate Bridge E) Williamstown.

Water physicochemical parameters were measured (pH, electrical conductivity (EC), temperature and dissolved oxygen (DO)) on each sampling event using a YSI 556 sonde (Xylem Analytics Australia Pty Ltd, Hemmant, Queensland) (Table 2.1). The EC increased along the Yarra River, from an average of 188 µS cm^{-2}± 11 µS cm^{-2} at Dights Falls to 45,637 µS cm^{-2} ± 1,655 µS cm^{-2} at the Westgate Bridge, the EC at the marine site at Williamstown had an average EC of 51,962 µS cm^{-2} ± 849 µS cm^{-2}.

Table 2.1Physico-chemical measurements for each site over the 12 month experimental period. Values show means and standard errors of the mean.

Site	**pH**	**EC (µS cm^{-2})**	**Temperature (^{o}C)**	**DO (%)**
Dights Falls	7.4 ± 0.2	188 ± 11	12.4 ± 1.9	92.7 ± 1.0
MacRobertson Bridge	7.5 ± 0.2	5,849 ± 655	12.6 ± 1.8	103.8 ± 4.7
Federation Square	7.5 ± 0.1	13,727 ± 2140	13.0 ± 1.7	96.9 ± 1.7
Westgate Bridge	7.9 ± 0.05	45,637 ± 1655	12.2 ± 0.4	103.8 ± 1.8
Williamstown	8.0 ± 0.2	51,962 ± 849	13.7 ± 1.1	111.4 ± 3.8

EC= Electrical conductivity, DO= Dissolved oxygen

2.3 Sample preparation

The substrates used were polylactic acid (PLA) coffee cup lids purchased from NatureWorks Ingeo®, Naarden, Netherlands and polypropylene (PP) take-away containers purchased from Alpha Packaging® Sydney, Australia. Glass microscope slides, used as a non-plastic control substrate, were purchased from Esco Scientific, Horsham, USA. PLA coffee cup lids and PP take-away container lids were cut using a 20 W laser system (Universal ® Laser Systems, Scottsdale, USA), with total areas of 54.7 cm^2 and 73.0 cm^2, respectively. The PP and PLA substrates were 0.6 mm and 0.4 mm thick, respectively. Glass slides had a surface area of 27.5 cm^2. Custom made individual holders for the glass slides were 3D printedfrom

acrylonitrile butadiene styrene (ABS) plastic by the RMIT University Advanced Manufacturing Precinct (Figure 2.3).

Figure 2.3 3D printed plastic glass slide holder. Scale bar represents 1 cm.

Samples were threaded onto nylon fishing line and strung across the frames in a random order with plastic straw spaces in between each sample to prevent them coming into contact with each other. Sample frames were buoyant in water and consisted of PVC frames (60 cm by 40 cm) (Figure 2.4). In total, over 900 samples were deployed, enough for triplicate samples for each of the time points (see below) and from each sample location with some extra samples to cover for sample loss during the experiment. The threaded substrates were sterilisedimmediatelybefore deployment by being wiped down with 70% ethanol.

Figure 2.4 Sample frame with substrates strung. Substrates had been extensively biofouled.

2.4 Sample collection and labelling regime

On June 25th, 2015 the samples were deployed with sampling events outlined in Table 2.2. Water samples were collected in sterile 500 mL glass Schott bottles on Day 1, Day 7 and Day 28 of sampling. Sample collection involved cutting one of the fishing lines from the sample frames and sliding the substrates into a bucket of water collected from the same location. Substrates intended for microbial analysis and microscopy were then slotted in racks or wrapped in ethanol rinsed aluminium foil. Substrates intended for chemical analysis were wrapped in acetone rinsed aluminium foil and kept in plastic sandwich bags. Substrates intended for structural tests were placed directly into plastic sandwich bags. All samples were stored on ice and transported back to the laboratory. All samples were then kept at -80°C.

Table 2.2Timing and dates of sampling

Sample event	Time from deployment	Date of sampling
0	/	25/06/15
1	1 Day	26/06/15
2	4 Days	29/06/15
3	7 Days	02/07/15
4	14 Days	09/07/15
5	28 Days	23/07/15
6	3 Months	24/09/15
7	6 Months	17/12/15
8	9 Months	06/04/16
9	12 Months	26/06/16

Due to sample loss caused by the nylon fishing lines breaking, samples were only collected from the Westgate Bridge site up to and including the third month of sampling, from Federation Square sites up to and including the sixth month of sampling, and from MacRobertson Bridge and Williamstown up to and including the ninth month. Samples were labelled to indicate the sample location, substrate type and sampling date. The location codes were Dights Falls (S1), MacRobertson Bridge (S2), Federation Square (S3), Westgate Bridge (S4) and Williamstown (S5). The substrate codes were polypropylene (PP), polylactic acid (L) and glass (G), the letter code was followed by a number indicating which replicate it was. The date codes were D for day and M for month. Using S1L3D28 as an example; Site 1, PLA replicate #3, Day 28.

Chapter 3: Variation inphysical and chemical properties of polypropylene and polylactic acid plastics following exposure in natural aquatic conditions

3.1 Introduction

Polylactic acid (PLA), also known as polylactide, is considered to be both a bioplastic and a biodegradable plastic (Gupta and Kumar, 2007, Madhavan Nampoothiri *et al.*, 2010). PLA is oneof the most commonly produced bioplastics, after biologically derived poly(ethylene terephthalate) (bio-PET),biologically derived polyamide (bio-PA) and biologically derived polyethylene (bio-PE) (European Bioplastics, 2017). The monomer of PLA, lactic acid (2-hydroxypropionic acid) can be created as a result of the fermentation of starchy feed-stocks such as corn- wheat- and potato- starches by several strains of *Lactobacillusspp.*(John *et al.*, 2008), which then undergoes a polycondensation reaction to form PLA. Bio-PET and bio-PE are both derived from mono-ethylene glycol (bio-MEG) which is also produced from plant sugars (Tsiropoulos *et al.*, 2015), while bio-PA is produced from castor oil (Kuciel *et al.*, 2012). Neither bio-PET nor bio-PE arereadily biodegradable under natural conditions, such as those found in aquatic environments; however, bio-PA is biodegradable in natural aquatic environments(Emadian *et al.*, 2017).

In natural environments,one of the main pathways for the degradation of PLA is photo-oxidative degradation (POD). In a process similar to that used for production of polyolefins such as polyethylene (PE) and polypropylene (PP), when PLAundergoes processing such as via extruding and moulding, chromophoric groups such as additional carbonyl groups are generated that are able to facilitate POD (Sin *et al.*, 2012)(see Section 1.5.1). The second most common abiotic degradation pathway of PLA in natural environments is via hydrolysis of the ester bonds of the PLA molecule (Grizzi *et al.*, 1995). Rates of hydrolysis of PLA are

affected by the degree to which the PLA structure is either crystalline or amorphous(Kale *et al.*, 2006), with hydrolysis more readily occurring in the amorphous regions. During the process of hydrolysis, chain scission occurs thereby reducing the molecular weight of the PLA molecule (Gewert *et al.*, 2015). Smaller water soluble oligomers also form and may migrate out of the PLA matrix, further lowering the molecular weight of the PLA molecules (Sin *et al.*, 2012).As hydrolysis of PLA occurs more readily in the amorphous regions, over time, the proportion of crystalline regions increases, leading to a decrease in the rate of degradation due to hydrolysis (Karamanlioglu *et al.*, 2017). Higher crystallinity of polymers is also correlated with higher tensile strength, partially due to the polymer chains no longer being able to slide across each other (Kong and Hay, 2002, Yu *et al.*, 2012).

The potential for biodegradation of plastics to occur has been known since the 1970s when wood-rot fungi were shown to degrade petroleum-based plastics under laboratory conditions(Albertsson, 1978). However, such degradation was found to be a slow process with a mass loss of only 0.5% for polyethylene recorded after 2 years in experiments using liquid media (Albertsson, 1978). In aquatic environments, plastic degrading microorganisms are rare, with only a few organisms being isolated directly from seawater that have been reported to be able to degrade plastics (see Section 1.5.2). Recently, a bacterium *Ideonella sakaiensis* 201-F6 capable of utilising low crystalline PET as its sole carbon source was isolated from marine sediments (Yoshida *et al.*, 2016). However, the ability for *I. sakaiensis* to utilise PET under natural aquatic conditions has not yet been demonstrated. Microorganisms in plastic biofilms may also degrade plastics via direct degradation of the plastic additives, and contribute to deterioration via penetration by fungal hyphae (Flemming, 1998, Rummel *et al.*, 2017).The main enzymes involved with the biodegradation of heteroatom plastics are hydrolases and esterases (Karamanlioglu *et al.*, 2017, Pathak and Navneet, 2017). Additional ezymes associated with PLA biodegradation include lipases,

proteases and cuninase-like-enzymes (Karamanlioglu *et al.*, 2017). Hydrolases and esterases are also involved with the biodegradation of polyolefin plastics such as PP and PE, after an intial abiotic degradation step has occurred such as via POD (Pathak and Navneet, 2017). In addition, alkane monooxygenase (AlkB) has also been associated with the biodegadation of low-molecular weight polyethylene (LMWPE) from bacteria isolated from oil contaminated soil (Jin and Kim, 2017).Alkane monooxygenases are well known for converting linear hydrocarbons into alcohols, aldehydes, carboxylic acids, andepoxides(Van Beilen and Funhoff, 2007).

Molecular changes to the polymers can be assessed using techniques such as water contact angle analysis which may be used as proxy to determine changes in the surface chemistry of the polymer(Maeda *et al.*, 1997); FTIR analysis which allows for specific changes in chemical bonds to be determined (Tabasi and Ajji, 2015); or molecular weight analysis using gel permeation chromatography (GPC) which may indicate chain scission of the polymer backbone(Martin *et al.*, 2014). Item weight loss is used to indicate mineralisation of the polymer or deteriation/fragmentation (Orhan *et al.*, 2004); however, changes in item weight are often not significant in plastic degradation studies (Lucas *et al.*, 2008). Changes to the tensile strength of a polymer may also indicate that chain scission has occurred (Lucas *et al.*, 2008). Tensile strength analysis is comparatively cheaper and less difficult to conduct than spectrometric analyses such as FTIR, but does not give insights into the specific chemical changes in polymer structure (Lucas *et al.*, 2008).

Previous studies investigating abiotic and biotic degradation of plastics have utilised a wide range ofthe above described techniques to assess degradation (Table 3.1), including measuringchanges to water contact angles (Maeda *et al.*, 1997, Nauendorf *et al.*, 2016), weight loss of the sample (Shogren *et al.*, 2003), molecular weight loss (Rudeekit *et al.*, 2008), changes in tensile strength (Rudnik and Briassoulis, 2011), biogas production (Kolstad

et al., 2012), pH changes in bioreactor mediums (Sikorska *et al.*, 2018), crystallinity (Gonzalez *et al.*, 1999) and Fourier transform infrared (FTIR) spectra (Torres-Huerta *et al.*, 2014, Tabasi and Ajji, 2015).Several of these studies were conducted at elevated temperatures (Gonzalez *et al.*, 1999, Bonhomme *et al.*, 2003, Ahn *et al.*, 2011, Kolstad *et al.*, 2012), which are not environmentally relevant when trying to determine the degradation of plastics in terrestrial and aquatic environments. These previous studies have also used different plastic structural types such as powders (Kunioka *et al.*, 2006), microspheres (Gonzalez *et al.*, 1999), films (Sikorska *et al.*, 2018) and solid objects (Ahn *et al.*, 2011). These methodological differences between studies make direct comparisons difficult due to these confounding factors. Moreover, research to assess the biodegradability of plastics is severely constrained due to the lack of consistent standards and procedures (Harrison *et al.*, 2018).

Table 3.1Examples of the various degradation test methods and procedures used to investigate degradation and deterioration of petroleum-based plastics and bioplastics.

Degradation test(s)	**Polymer/ Composite type**	**Period of trial and environment**	**Amount of degradation**	**Reference**
Water contact angle	COMP* and PE bags	98 days in sediment microcosms	Water contact angle for both COMP and PE bags decreased by at least 25^{o} by 98 days.	(Nauendorf *et al.*, 2016)
Tensile strength	PLA	11 months buried in soil outside.	After 1 month samples were too brittle to undergo tensile strength testing.	(Rudnik and Briassoulis, 2011)
Tensile strength	Mater-Bi^ and PE bags	24 months in aquaria	Max Load at break reduced by 66% by Month 24 for the Mater-Bi bags. Max Load at break reduced increased for PE bags by ~50% by Month 24.	(Tosin *et al.*, 2012)
Tensile strength	Mater-Bi and PE bags	33 days in seawater	Decrease in Max Load at breaking point. However, statistical significance was not tested.	(Eich *et al.*, 2015)
Weight loss and tensile strength	PLA	1 year in outdoor soil plots.	~0% $year^{-1}$ weight loss. No change in tensile strength.	(Shogren *et al.*, 2003)
Weight loss and tensile strength	PLA/PHEE	1 year in outdoor soil plots.	4-50% weight $lossyear^{-1}$. No change in tensile strength.	(Shogren *et al.*, 2003)
Molecular weight	PLA	15 months in landfill.	PLA sheets began disintegrating within 6 months.	(Rudeekit *et al.*, 2008)
Molecular weight	PLA	34 days in a compost system.	PLA sheets could not be found by Day 34.	(Rudeekit *et al.*, 2008)
Molecular weight	PLA and PEA	45 days in seawater microcosm	PLA: ~0% molecular weight loss after 45 days, PEA: ~15% molecular weight loss after 45 days.	(Martin *et al.*, 2014)
CO_2 production	PLA/poultry feather/ starch	60 days in compost bioreactors kept at 58 ^{o}C.	53 ± 2% degradation within 60 days.	(Ahn *et al.*, 2011)
Biogas production	PLA	390 days in anaerobic soils at 21 ^{o}C.	~0% biogas production from crystalline PLA after 390 days.	(Kolstad *et al.*, 2012)
pH of the medium	PLA/PHB	180 days in distilled water at 70 ^{o}C.	pH decreased from 6.78 to 2.36 after 56 days due to release of acidic products into the medium.	(Sikorska *et al.*, 2018)

Crystallinity	PLA	37 ^{o}C in buffer solution (Titrisol, pH = 7) for up to 247 days	PLA crystallinity reached a constant 53 % by Day 70 from an intial crystallinity of 0 %.	(Gonzalez *et al.*, 1999)
FTIR	PE	40 ^{o}C up to 1,600 h in a hot air oven	Carbonyl groups formed after 400 h at 40^{o}C.	(Bonhomme *et al.*, 2003)
FTIR	PET/PLA and PET/ chitosan	1,200 h in accelerated weathering chamber	FTIR bands characteristic of ester bonds became weak, indicating chain scission of the C─O bonds.	(Torres-Huerta *et al.*, 2014)
ATR-FTIR	PLA/PBAT	45 days in a composting system	Bonds indicative of lactic acid developed by Day 6, suggesting autocatalytic hydrolysis of the PLA.	(Tabasi and Ajji, 2015)

FTIR= Fourier transform infrared spectroscopy,ATR-FTIR= Attenuated total reflectionFourier transform infrared spectroscopy,PLA= polylactic acid, PEA= Polyesteracetal copolymers of 1,3-dioxolan-4-one (DOX) with PLA, PHEE= poly(hydroxyester-ether),PHB= polyhydroxybutyrate, PE= polyethylene, PBAT= Butylene adipate-coterephthalate.

*COMP comprised of >50% biodegradable polyester, >20% corn starch and a proprietary component (Nauendorf *et al.*, 2016).

^Mater-Bi is a copolyester derived from vegetable oils and plant starches (Tosin *et al.*, 2012).

*In vitro*aquatic degradation studies of petroleum-based plastics and bioplastics have demonstrated that POD and hydrolysis are important mechanisms for degradation of plastics(Tosin *et al.*, 2012).However,due to the experimental designof the *in vitro*studies,effects such as wave action, or development and succession of microbial biofilm communities were not able to be taken into accountin these studies (Tosin *et al.*, 2012). For example, an *in vitro* study of the degradation of PLA involved maintaining PLA films in baths of seawater at 20 ^{o}C and 40 ^{o}C for a 3 month period (Le Duigou *et al.*, 2009). Molecular weight analysis using GPC showed that molecular weight of the PLA reduced in the 40 ^{o}C treatment by 48 % and in the 20 ^{o}C treatment only by 14 %, indicating that

hydrolysis of the PLA had occurredin both treatments (while also highlighting the impact of elevated, non-environmentally relevant temperatures to polymer degradation), althoughthe Young's modulus (a measurement of elascity assessing the ratio of stress (σ) to strain (Christopher and James, 2009)) of the PLA did not significantly change over the 3 month experimental period for either of the treatment types. However, in the studies of PLA in tanks the strain at break increased by 335 % for the 20 ^{o}C treatment and fell by 48 % for the 40 ^{o}C treatment;whilst for both treatments, the maximum stress fell by >20 %. The authors postulated that the low sensitivity of the Young's modulus could be a result of structural reorganisation (recrystallization) of the polymers. Another*in vitro*study investigating degradation of PE, exposed PE pellets in batch reactors either containing artificial seawater or deionised water under a UV lamp (operating at 254 nm and with an intensity of 400 mW cm^{-2}) for eight weeks (Da Costa *et al.*, 2018). By the conclusion of the experiment, peaks in the FTIR spectra in the region of 1,600 cm^{-1}-1,630 cm^{-1} developed in both treatments, corresponding to formation of carbonyl groups indicating that oxidation of the PE had occurred. Moreover, the peaks indicative of carbonyl groups were stronger for the artificial seawater treatment when compared to the deionised water treatment. Therefore, there may be hitherto unknown effect of salinity on the structural and morphological properties of PE (Da Costa *et al.*, 2018).

To date, there has been only one study of the degradation of PLA under *in-situ* aquatic conditions (Tsuji and Suzuyoshi, 2002),by comparison there have been many degradation studies of PLA in compost systems (Emadian *et al.*, 2017). This lack of research in aquatic environments is a critical gap in knowledge as it can be assumed that PLA that is littered is likely to enter aquatic environments as opposed to composting systems. For example, in composting systems, PLA has been found to degrade over a relatively short amount of time (80% in 50 days), measured by the production of CO_2(Kunioka *et al.*, 2006). However, these

results have been largely variable, with degradation rates ranging from 13% in 60 daysto 100% in 28 days, measured by the production of CO_2 and sample mass loss, respectively (Ahn *et al.*, 2011, Arrieta *et al.*, 2014).

The previousaquatic *in situ*study of PLA degradation by Tsuji and Suzuyoshi (2002)was conducted over a relatively short period of time of only five weeks in seawater using thin films (25 μm - 50 μm). Over this five week period there were no significant changes in the molecular weight of either amorphous- PLA or crystalline- PLA (Tsuji and Suzuyoshi, 2002). However, the tensile strength of both PLA films types reached 0 % (residual elongation at break) and the weight loss of the PLA substrate was between 30% - 70%. The authors suggested that the results indicated that physical processes such as wave action are major disintergration/fragmentation pathways for breakdown of PLA films in aquatic environments (Tsuji and Suzuyoshi, 2002). There have been several studies investigating*in situ* aquatic degradation of other types of plastics such as the biodegradable polycaprolactones (PCL) and polyhydroxyalkanoates (PHA) (Table 3.2). PCL is a polyester and is produced from condensation and ring-opening polymerisation (ROP) reactions of6-hydroxycaproic (6-hydroxyhexanoic) acid and of Ɛ-caprolactone, respectively (Labet and Thielemans, 2009).PCL is often used for medical purposes such as tissue scaffolding (Malikmammadov *et al.*, 2018), and is readily degradable under aquatic conditions (Rutkowska *et al.*, 1998). For discussion of PHA production its and properties refer to Section 1.2. Similar to the studies described in Table 3.1, the *in situ*plastic degradation studies have each used different experimental procedures and experimental designs, although tensile strength test were the mostly commonly used measurement, making direct comparisons between studies difficult due to the cofounding factors. The tensile strength of poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV) (Imam et al., 1999), polycaprolactone (PCL) (Tsuji and Suzuyoshi, 2002), poly(R-3-hydroxybutyrate) (R-PHB) (Tsuji and Suzuyoshi, 2002) and PLA (Tsuji and

Suzuyoshi, 2002)substrates have been found to decrease to 0 % by 5 weeks of exposure in aquatic environments.

Table 3.2*In situ* plastic degradation studies in aquatic environments.

Polymer type	Period of trial and environment	Analytical technique	Amount of degradation	Reference
PHA	254 days in a freshwater lake, at depth of 85 m.	Weight loss	10-20 mg d^{-1} of weight loss.	(Brandl and Püchner, 1991)
PCL	2 months in seawater in the Baltic Sea.	Tensile strength	Destroyed by week 8.	(Rutkowska *et al.*, 1998)
PHBV	1 year in seawater in Puerto Pico.	Tensile strength	Destroyed by month 12.	(Imam *et al.*, 1999)
PCL, R-PHB and PLA	5 weeks in seawater in Japan.	Gravimetry and tensile strength	By 5 weeks weight losses were; PCL: ~30 %; R-PHB: ~65 % and PLA ~30 %-70 %. Tensile strength of all three polymer types approached 0 % by week 5.	(Tsuji and Suzuyoshi, 2002)
PC	3 months in seawater in India	FTIR	Increase in the formation of substituted alkyl phenols on the PC.	(Artham *et al.*, 2009)
PCL, PHBV and PBS	1 year seawater in Japan, at a depth of 350 m.	Tensile strength	Tensile strength reduced to 0 % for PCL (9 months), PHBV (3 months). 90 % of tensile strength of PBS remained after 12 months.	(Sekiguchi *et al.*, 2011)
PHA	160 days in seawater in Thailand	GPC	Molecular weight loss ranged between 16 % - 26 % after 160 days.	(Volova *et al.*, 2011)
PE, PP and PS	32 weeks in a saltmarsh,	Fragmentation	Microplastics were detected after 8 week samples and increased in the 32 week samples for all three plastic types.	(Weinstein *et al.*, 2016)
PE and Mater-Bi carrier bags*	12 months in seawater , at depths betwwen 25 m and 36 m.	Tensile strength	Statistically significant decrease in tensile strength of Mater-Bi® bags of ~6 N, while no statistically significant decrease in the tensile strength of the PE bags.	(Pauli *et al.*, 2017)

PHA = Polyhydroxyalkanoate, PCL = Polycaprolactone,PHBV = Poly(β-hydroxybutyrate-co-β-hydroxyvalerate), R-PHB = Poly(R-3-hydroxybutyrate), PLA = Polylactic acid, PC = Polycarbonate, PE = Polyethylene, PP = Polypropylene, PS = Polystyrene, FTIR= Fourier Transform Infrared spectroscopy, PBS = Poly(butyrene succinate),GPC= Gel permeation chromatography

*Mater-Bi is a copolyester derived from vegetable oils and plant starches.

The aim of this current study was to compare variation in the structural properties of PP and PLA, via analysis of surface hydrophobicity, tensile strength, crystallinity and chemical structure over a 12-month exposure experiment in surface waters at five locations along a freshwater-marine continuum. The plastics chosen were rigid single-use consumer items [Take-away containers (PP) and coffee cup lids (PLA)]and which are likely to enter the environment as litter. To assess potential surface chemical changes of the plastics, surface hydrophobicity (water contact angle (WCA)) and Fourier Transform Infrared (FTIR) spectroscopy measurements were taken. To assess potential internal physical changes of the plastics, tensile strength and X-ray diffraction (XRD) spectra (polymer crystalinity) were measured. Biofilm development on the plastic surfaces was also assessed. It was anticipated that PLA substrates would lose structural integrity more rapidly than PP substrates.

3.2 Methods

3.2.1 Plastic surface hydrophobicity analysis via water contact angle (WCA) measurements

Experimental design of the exposure study was previously described in Chapter 2.

After collection (see Chapter 2), plastic substrates were washed using a sponge, rinsed with deionised water and allowed to dry,to remove biofilms and expose the plastic surface. An example of an unwashed and a washed PP coupon is shown in Figure 3.1. WCA were then analysed using a contact angle analyser, model OCA 20 (DataPhysics Instruments GmbH, Filderstadt, Germany) using the sessile drop method(Drelich, 2013). Milli-Q water(1 μL) was applied to the plastic surface; the eclipse from the droplet was used to measure the WCA. Areas on the plastic surface from which the biofilm/pigments were not able to be removed were avoided. A wetting effect occurred if the water droplet was placed on the biofilm/pigments patches (data not shown). Triplicate measurements were taken for three coupons per sampling event.

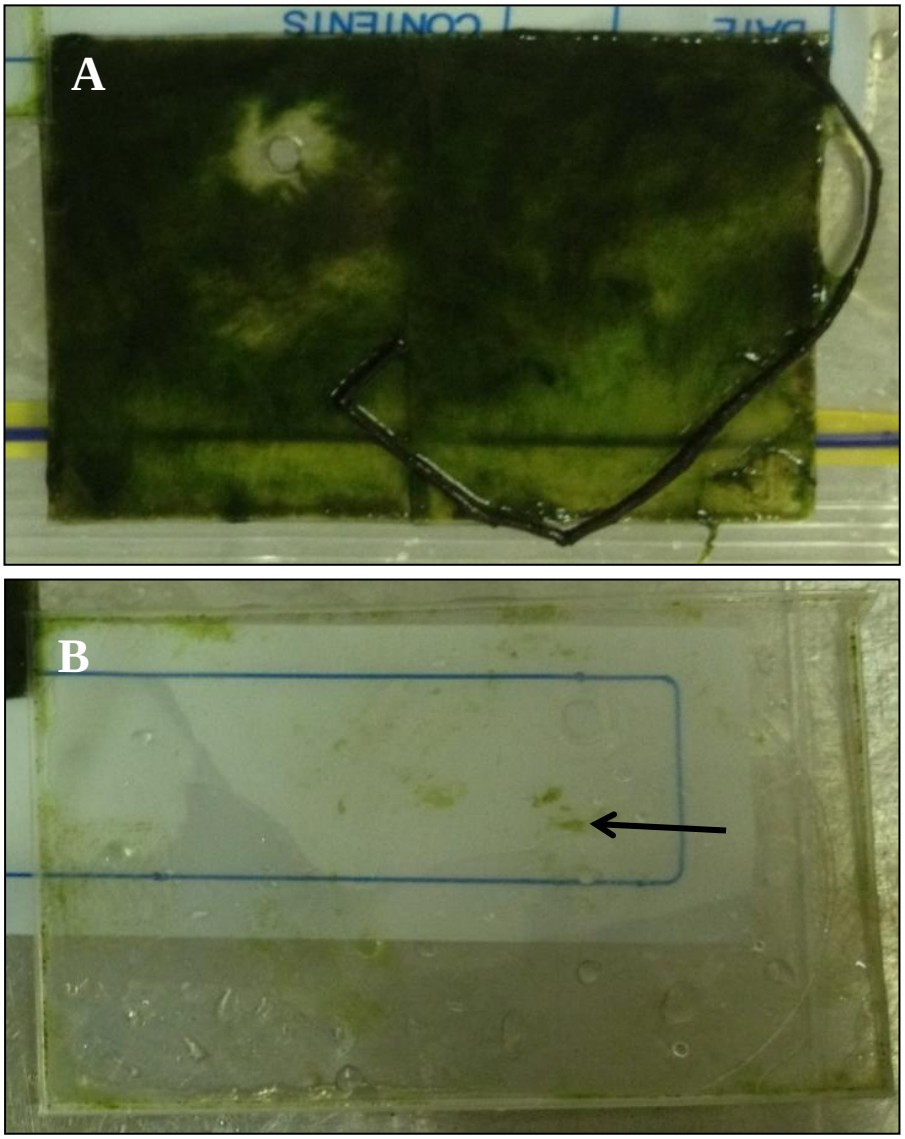


Figure 3.1 A polypropylene substrate coupon recovered from Site 3 at Month 3. A) Uncleaned coupon B) Coupon after washing to remove biofilm. The arrow indicates an area in which the biofilm/pigments were not able to be removed (such areas were avoided for water contact angle determinations).

3.2.2 Plastic Universal Tensile Strength analysis

The dimensions of the commercial PP and PLA substrates used in this study weretoo thin(thicknesses of the PP and PLA substrates were 0.6 mm and the 0.4 mm, respectively)for analysis either using international standards ASTM D638 (suitable for polymers with thicknesses between 1 mm – 14 mm) or ASTM D882 (suitable for polymer films with thicknesses <1 mm) for determination of Universal tensile strength (ASTM international, 2012, ASTM international, 2014). Consequently, a hybrid method was developed and used.Briefly, polymer coupons were washed with luke-warm water and wiped with a cloth to remove any biofilm from the surface. Coupons were then cut into 'dog bone' shapes as detailed in ASTM D638 using a 20 W laser system (Universal ® Laser Systems, Scottsdale, USA) at 20% Power and 9% Speed for the PP samples and at 20% Power and 15% Speed for PLA samples. The dimensions were altered and are as detailed in Figure 3.2. All samples were strained at 1.5 mm min^{-1} using an Instron® Table Top Load Frame (Model 4465; Instron, Norwood, USA). All coupons were acclimatised to room temperature for a period of at least 48 h prior to testing. The measurements taken were the max load (N) and the Young's Modulus (ratio of stress/strain).

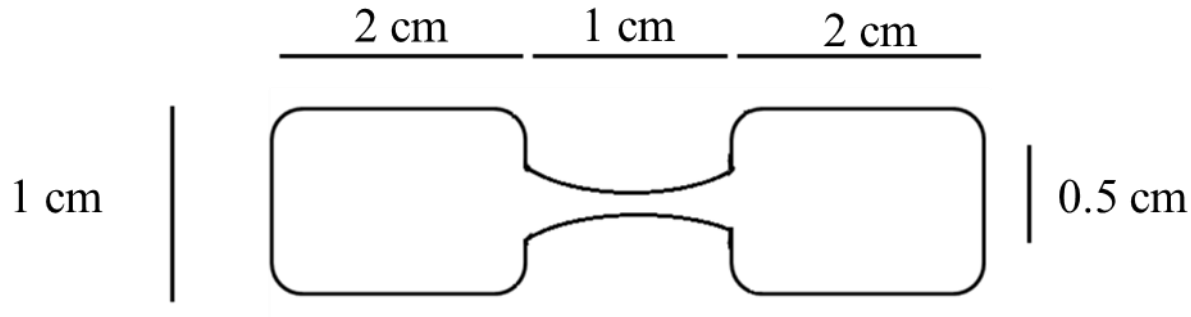


Figure 3.2 Dimensions for 'dog bone' coupon substrates used for universal tensile strength tests (not to scale).

3.2.3 Chemical structure and crystallinity analysis of polymer substrates

A random sample for each polymer coupon type (PP and PLA) from samples recovered from Day 1 and Month 6 from Dights Falls and Williamstown were chosen for analysis. Samples were prepared in a similar nature to the WCA samples (see Section 3.2.1), in addition 2 cm x 1 cm strips of the polymers were cut from the coupons.

3.2.3.1 Fourier transform infrared (FTIR) spectroscopy

A GladiATRTM (Pike Technologies, Madison, USA) was used in the range of 500 cm^{-1}–4,000 cm^{-1} (wavenumber) to investigate the chemical structure of the polymer substrates using methods outline by Truskewycz *et al.* (2018). The resolution was 4 cm^{-1}. Software Spectrum ™ 10 (PerkinElmer, Waltham, USA) was used to process the spectra.

3.2.3.2 Polymer crystallinity analysis

Crystallinity of PLA and PP substrates was conducted via X-ray diffraction (XRD) (D4 Endeavor; Bruker, Billerica, USA) at room temperature using methods outline by Truskewycz *et al.* (2018). The instrument was equipped with a Cu Kα radiation source (wavelength 0.1542 nm) operating at 40 kV and 35 mA. All X-ray data was obtained in the *u-2u* locked-couple mode over a 2*u* interval of 10-90. The diffraction peaks were identified by the software Diffrac.Eva V4.2.1 (Bruker, Billerica, USA).

3.2.4 Scanning Electron Microscopy (SEM) imaging

Small pieces were cut from the polymer coupons(ranging between 0.5 cm^2 and 1.0 cm^2) using sterile scissors. These pieces were dried in ethanol baths in series from 50%, 60%, 80 %, 90 % to absolute ethanol. The samples were then mounted on metal stubs and coated in gold using a SPI-MODULE ™ Sputter Coater (SPI Supplies, West Chester, USA). Scanning electron microscopy (SEM) images were taken using a XL 30 microscope (Philips, Amsterdam, Netherlands).

3.2.5 Data analysis

The statistical software package SPSS (IBM SPSS Statistics version 21) was used for the statistical analysis of WCA and tensile strength data. Normality was tested using the Shapiro-Wilk test(Shapiro and Wilk, 1965). Significance (p) was <0.05, therefore normality was rejected. Hence, non-parametric tests were chosen; namely Wilcoxon Signed Rank Test(Wilcoxon, 1945) and Kruskal-Wallis pair-wise comparisons(Kruskal and Wallis, 1952). The Wilcoxon test was used to compare data between the start and end of the experimental period, while the Kruskal-Wallis analysis was used to compare samples between each pair of time points. For both tensile strength analysis and WCA analysis, results from the multiple sites were each combined for each time point as some replicates at some of the sites were lost from sites over the duration of the exposure experiment.

The decision was made *post hoc* to determine the chemical structure and degree of crystallinity of the different polymer substrate types.Stored samples from Month 6 were those available that had had the longest exposure time in the water when the decision was made to conduct the Fourier transform infrared (FTIR) and X-ray diffraction (XRD) analysis.

3.3 Results

3.3.1 Variation in water contact angles (WCA) of polymer substrates

Water contact angle (WCA) measurements for PP and PLA were taken as a proxy to indicate the surface hydrophobicity of the substrates (Figure 3.3). The initial WCA was higher for the PP substrates ($\Theta = 86.8^{o} \pm 8.8^{o}$) when compared to the initial WCA of the PLA substrates ($\Theta = 65.5^{o} \pm 7.2^{o}$). There was no significant difference based on the Wilcoxon Signed Rank Test ($P>0.05$) for either coupon type when comparing the WCA from samples from Day 1 with samples from Month 12. Kruskal-Wallis analysis was conducted to investigate variation in the WCA for each polymer between different sampling dates. Significant differences ($P < 0.05$) after Bonferroni corrections in the WCA for PLA substrates between the sampling dates was observed only between Month 3 and Month 9 with coupons at Month 9 having a significantly lower WCA than at Month 3 (Month 3 $\Theta = 73.6^{o} \pm 10.0^{o}$; Month 9 $\Theta = 59.5^{o} \pm 7.4^{o}$).

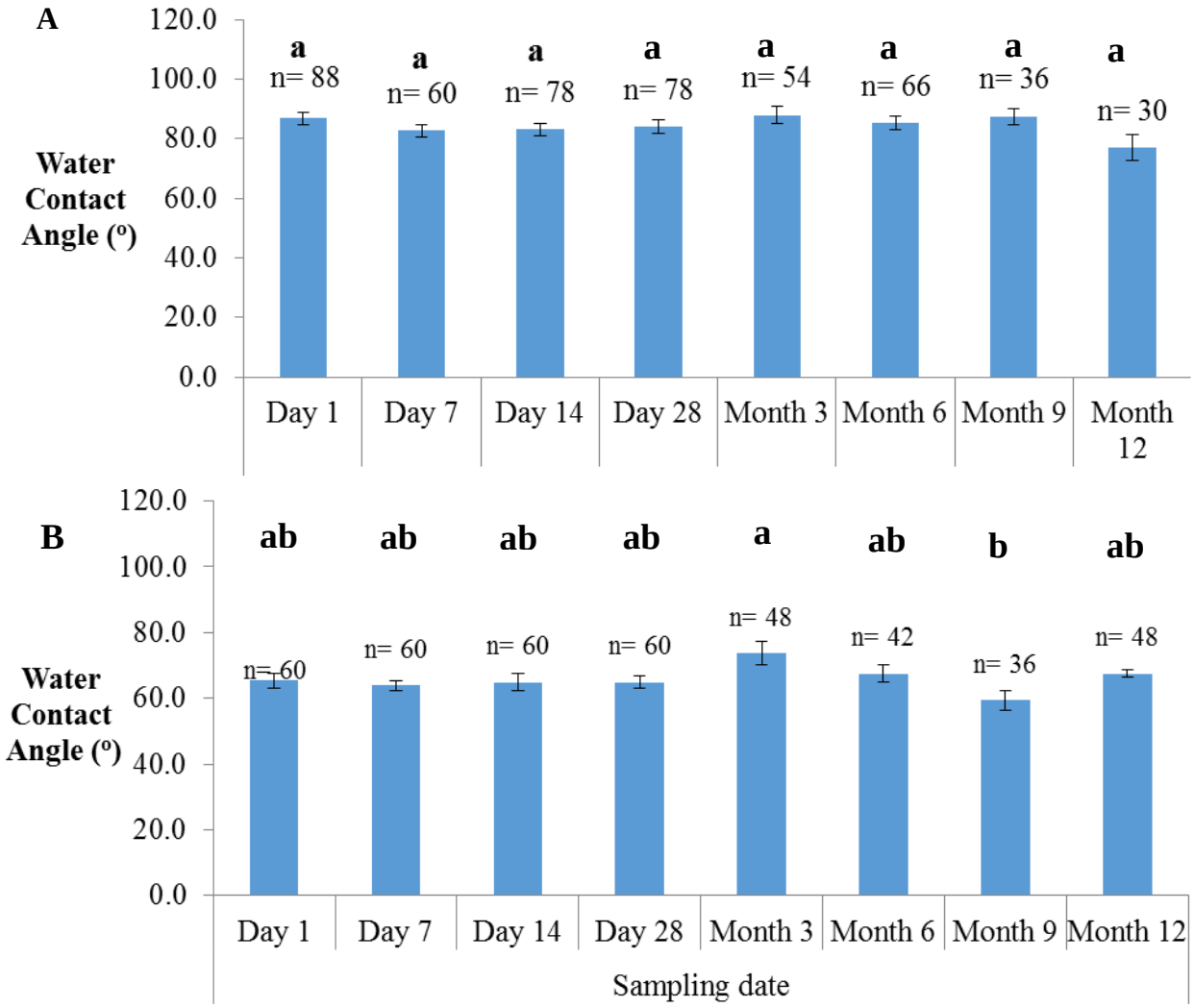


Figure 3.3Variation in water contact angle (WCA; indicating changes in surface hydrophobicity) of substrates exposed in surface waters for up to 12 months along the Yarra River, Melbourne.A)Polypropylene (PP) substrates;B) Polylactic acid (PLA) substrates. Samples from all sites were analysed together to increase replication for each time point. Error bars show the standard error of the mean. Sample sizes (number of measurements taken) are depicted at the top of each bar. Significant differences in the WCA ($P<0.05$) between sampling time points viaKruskal-Wallis analysis are depicted in lower case (a, b).

3.3.2 Variation in tensile strength of polymer substrates

Physical changes to the plastic substrates were assessed using the universal tensile strength test; measurements of Max Load at breaking point (Figure 3.4) and Young's Modulus (Figure 3.5). The PP substrates initially had a lower Max Load and Young's Modulus (39 N ± 2.0 N and 995 MPa ± 20 MPa, respectively) when compared to the Max Load and Young's Modulus of the PLA substrates (45 N ± 1.7 N and 1901 MPa ± 13 MPa, respectively).

There was a significant difference based on the Wilcoxon Signed Rank Tests($P<0.05$) for the PLA substrates when comparing the Max Load from samples from Day 1 (45 N ± 1.7 N) with samples from Month 12 (50 N ± 0.8 N)(Figure 3.4B). There was no significant change in the Young's Modulus from the beginning (Day 1) to the end (Month 12) of the experiment for either PP or PLA substrates ($P>0.05$) (Figure 3.5).

Kruskal-Wallis analysis was conducted to investigate variation in the polymer Max Load at breaking point and Young's Modulus for both substrate types between different sampling dates.The Max Load of the PP substrates was lower for the samples recovered on Day 1(39 N ± 2.0 N) and Month 6 (40.6 N ± 1.3 N) when compared to samples from Month 9(44.9 N ± 0.8 N) (Figure 3.4A). The Max Load of the PLA substrates was lower for the samples recovered on Day 1 (45 N ± 1.7 N) and Month 9 (44.1 N ± 1.5 N) when compared to samples from Month 12 (50 N ± 0.8 N)(Figure 3.4B). No significant differences in either coupon type in Young's Modulusbetween the individual sampling dates ($P>0.05$) were observed (Figure 3.5).

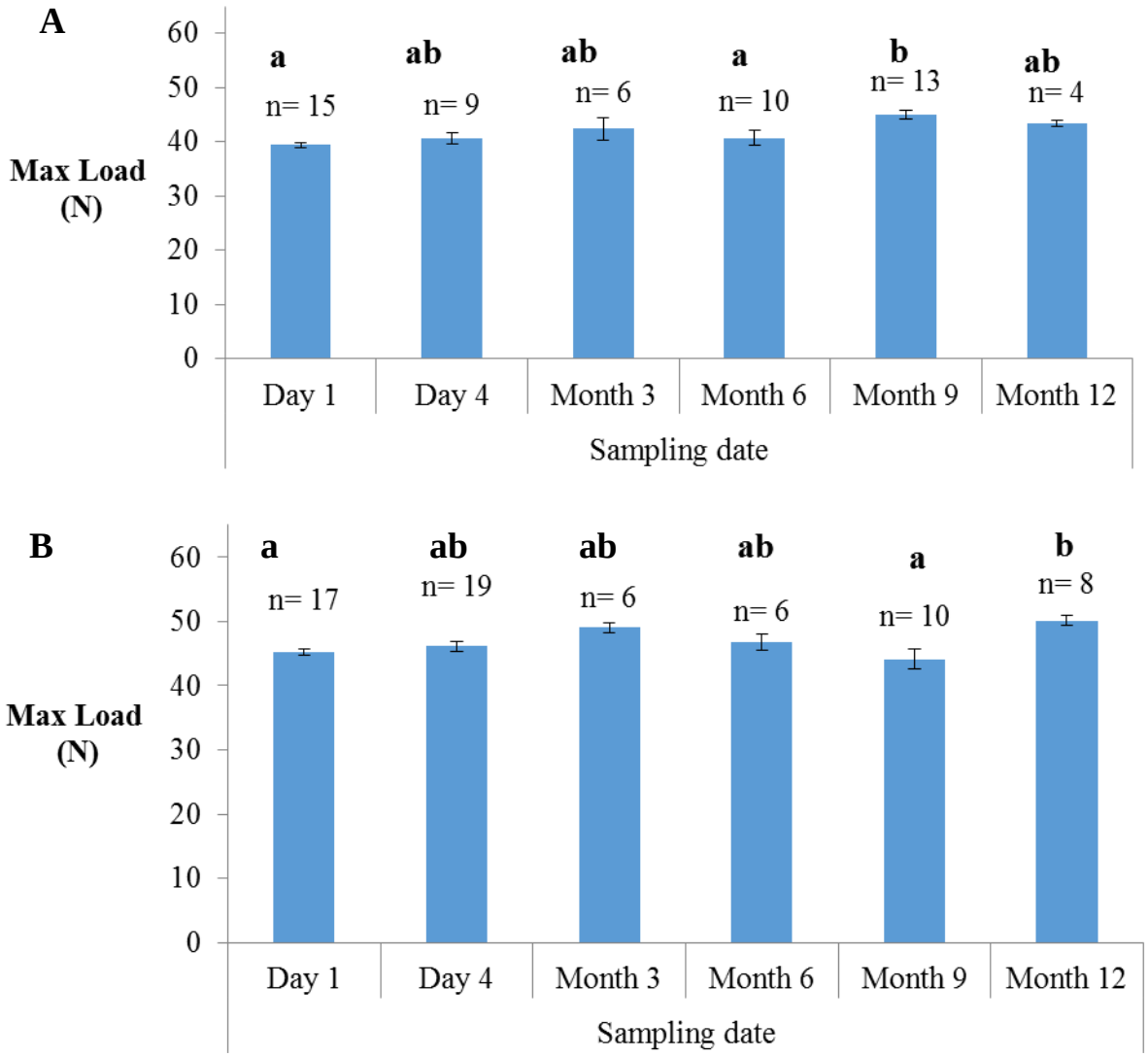


Figure 3.4Variation in the Max Load at breaking for plastic substrates exposed in surface waters for up to 12 months along the Yarra River, Melbourne. A)Polypropylene (PP) substratesB) Polylactic acid (PLA) substrates. Samples from all sites were analysed together to increase replication for each time point. Error bars show the standard error of the mean. Sample sizes (number of measurements taken)are depictedabove each bar. Significant differences in the Max Load ($P<0.05$) between sampling time points viaKruskal-Wallis analysis are depicted in lower case (a, b).

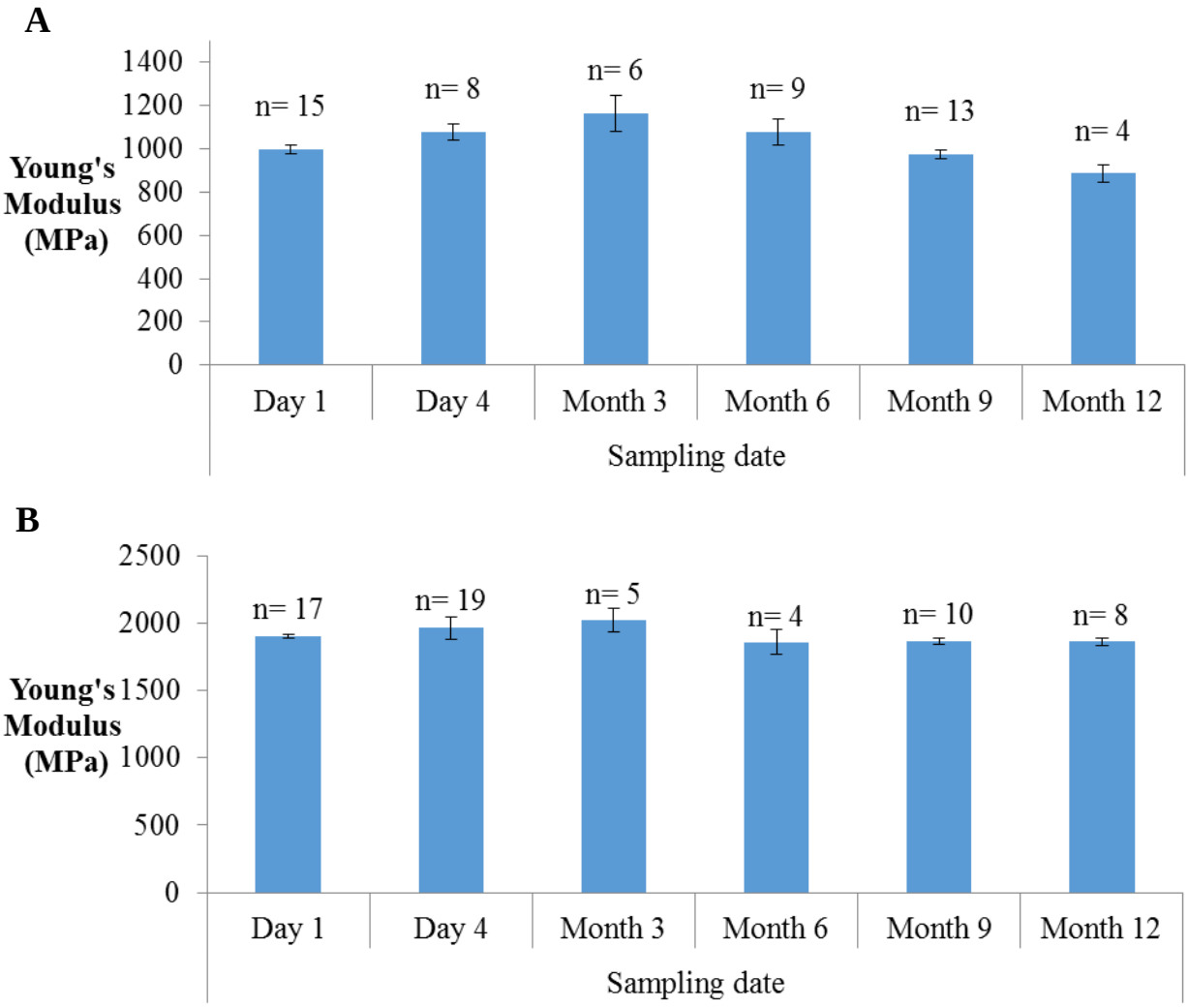


Figure 3.5Variation in the Young's Modulus for plastic substrates exposed in surface waters for up to 12 months along the Yarra River, Melbourne. A)Polypropylene (PP) substratesB) Polylactic acid (PLA) substrates. Samples from all sites were analysed together to increase replication for each time point. Error bars show the standard error of the mean. Sample sizes (number of measurements taken)are depicted above each bar. Significant differences in the Young's Modulus ($P<0.05$) between sampling time points viaKruskal-Wallis analysis were not observed.

3.3.3 Chemical structure and crystallinity analysis of polymer substrates

3.3.3.1 Crystallinity of polymer substrates

The crystallinity of individualPLA and PP substrates coupons, collected from Dights Falls and Williamstown from the Day 1 and Month 6 sampling events was determined using X-raydiffraction (XRD) (Table 3.3). XRD spectra are presented in *Appendix I*. PP substrates were initially more crystalline when compared to the PLA substrates (PP Day 1: 75.4 % and 76.8 %, PLA Day 1: 38.6 % and 40.0 %). The PLA substrate crystalinities increased by Month 6 to 48.1 % and 50.8 %, while the PP substrate crystalinities decreased to 69.9 % and 73.8%.

Table 3.3Polymer crystallinity at room temperature of polylactic acid (PLA) and polypropylene (PP) substrates based on X-ray diffraction (XRD) results

Substrate	Date	Location	Sample	Crystallinity percentage (%)
PLA	Day 1	Dights Falls	D1S1L	40.0
	Day 1	Williamstown	S1S5L	38.6
	Month 6	Dights Falls	M6S1L	50.8
	Month 6	Williamstown	M6S5L	48.1
PP	Day 1	Dights Falls	D1S1P	75.4
	Day 1	Williamstown	D1S5P	76.8
	Month 6	Dights Falls	M6S1P	73.8
	Month 6	Williamstown	M6S5P	69.9

3.3.3.2 Fourier transform infrared (FTIR) spectroscopy of polymer substrates

Individual samples of PP andPLA collected from Dights Falls and Williamstown from the Day 1 and Month 6 sampling events were analysed using Fourier transform infrared spectroscopy (FTIR)(Figure 3.6) (Samples from Month 6 were the longest exposed samples that were available). FTIR spectra from PP coupons (Figure 3.6A) had strong peaks in the regions of 2,949 cm^{-1}, 2,916 cm^{-1}, 2,855 cm^{-1}, 2,837 cm^{-1}, 1,452 cm^{-1} and 1,375 cm^{-1} which

Chapter 3: Variation in structural properties of plastic under natural aquatic conditions are indicative of various carbon-hydrogen bonds as well as weak peaks in the regions of 1,155 cm^{-1}, 997$cm^{-1}$and 972 cm^{-1} which are indicative of C=C bonds. FTIR spectra from PLA coupons (Figure 3.6B) had strong peaks in the regions of 1,745 cm^{-1}1,180 cm^{-1} and 1,041 cm^{-1}which are indicative of ester linkages and weak peaks in the regions 2,995 cm^{-1},2,945 cm^{-1}, 1,451$cm^{-1}$and 1,381 cm^{-1} which areindicative of various carbon-hydrogen bonds. No changes were seen in the FTIR spectra for either the PP or PLA coupon types between Day 1 and Month 6 from either the Dights Falls or Williamstown sample sites, indicating that chemical changes had notoccurred to either coupon type.

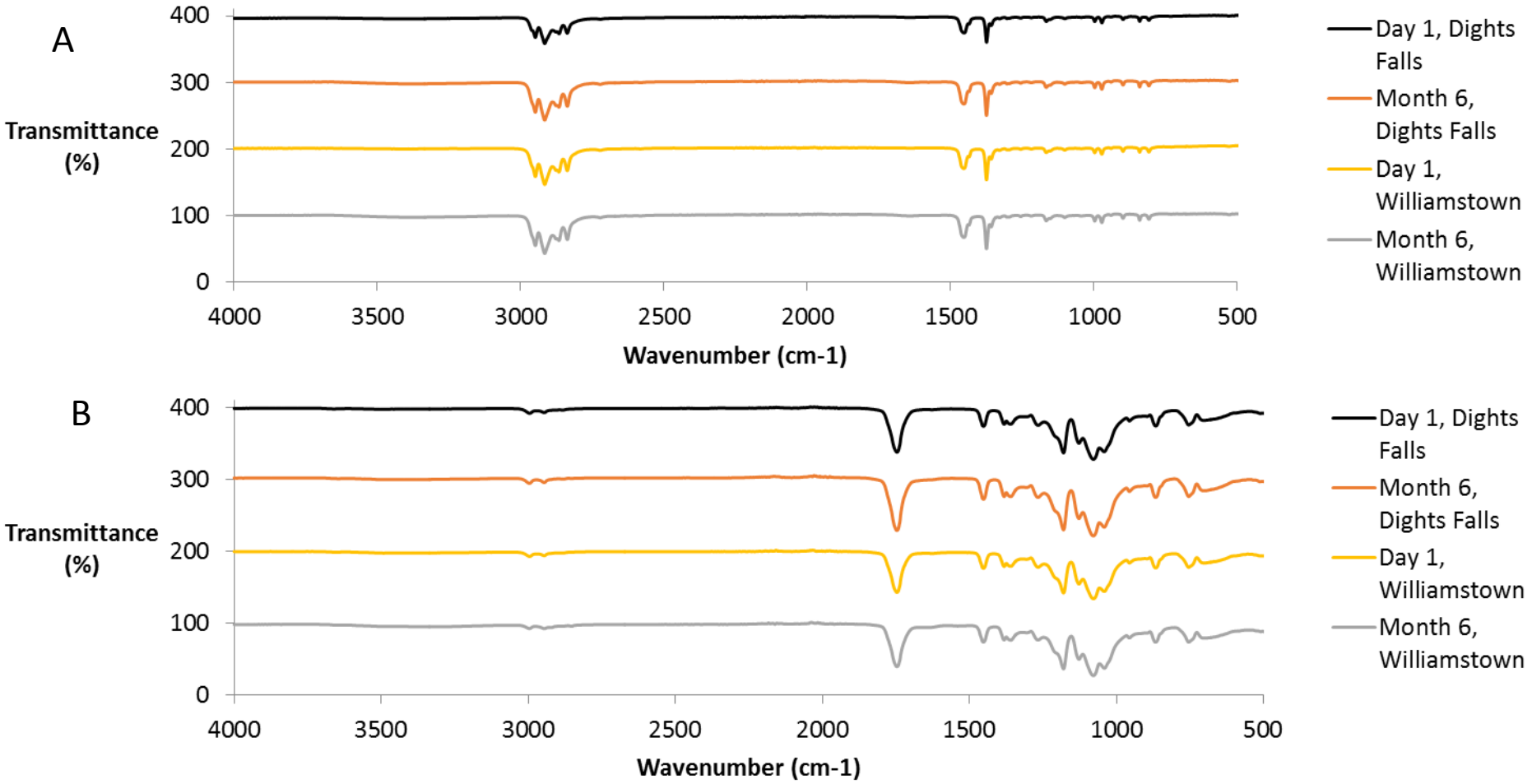


Figure 3.6Fourier-transform infrared (FTIR) transmission peaks of representative A) polypropylene (PP) and B) polylactic acid (PLA) coupons recovered from Dights Falls and Williamstown sites from Day 1 and Month 6 sampling dates.

3.3.4 Biofilm formation on polymer substrates after exposure in the Yarra River

SEM images were taken of both PP (Figure 3.7) andPLA (Figure 3.8)coupons at Dights Falls and Williamstown for Day 1 and Month 3 samples. By the third month of the experiment, both PP andPLAsubstrates had been extensively colonised and completely covered by complex microbial communities, comprising algae, diatoms and bacteria, and in some cases (Williamstown at Month 3 of PP) supporting the presence of herbivorous copepods (*Cyclops* sp.)(Figure 3.7D). Both PP and PLA coupons were extensively covered with algae by Month 3 at Dights Falls, and by bryozoa and mussels by Month 6 at Williamstown (Figure 3.9).

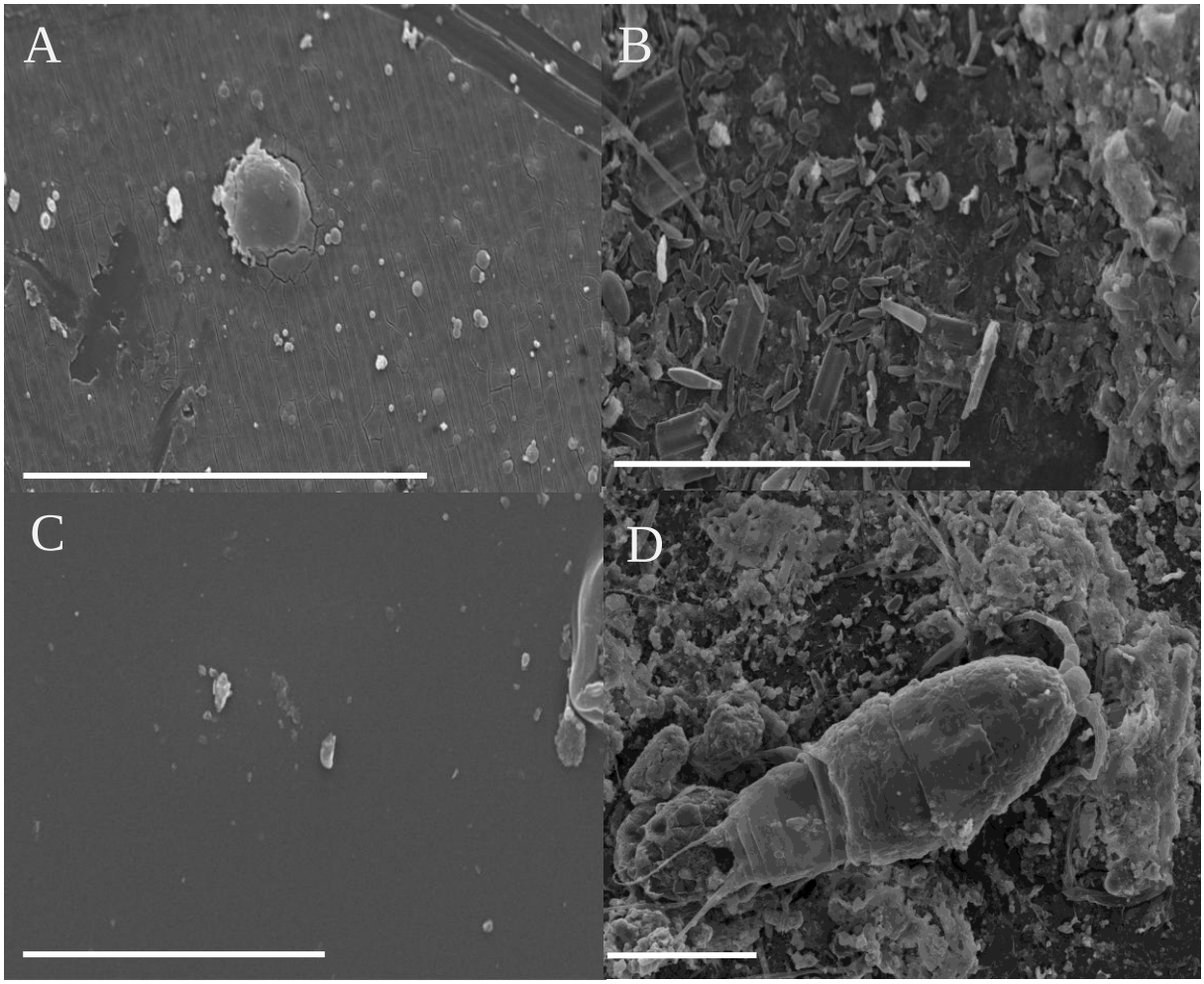


Figure 3.7Scanning electron microscope (SEM) images ofpolymer surfaces and biofilm formation onpolypropylene (PP) substrates from A) Dights Falls, Day 1 B) Dights Falls, Month 3 and C) Williamstown, Day 1 D) Williamstown, Month 3. Scale bars represent 250 μm.

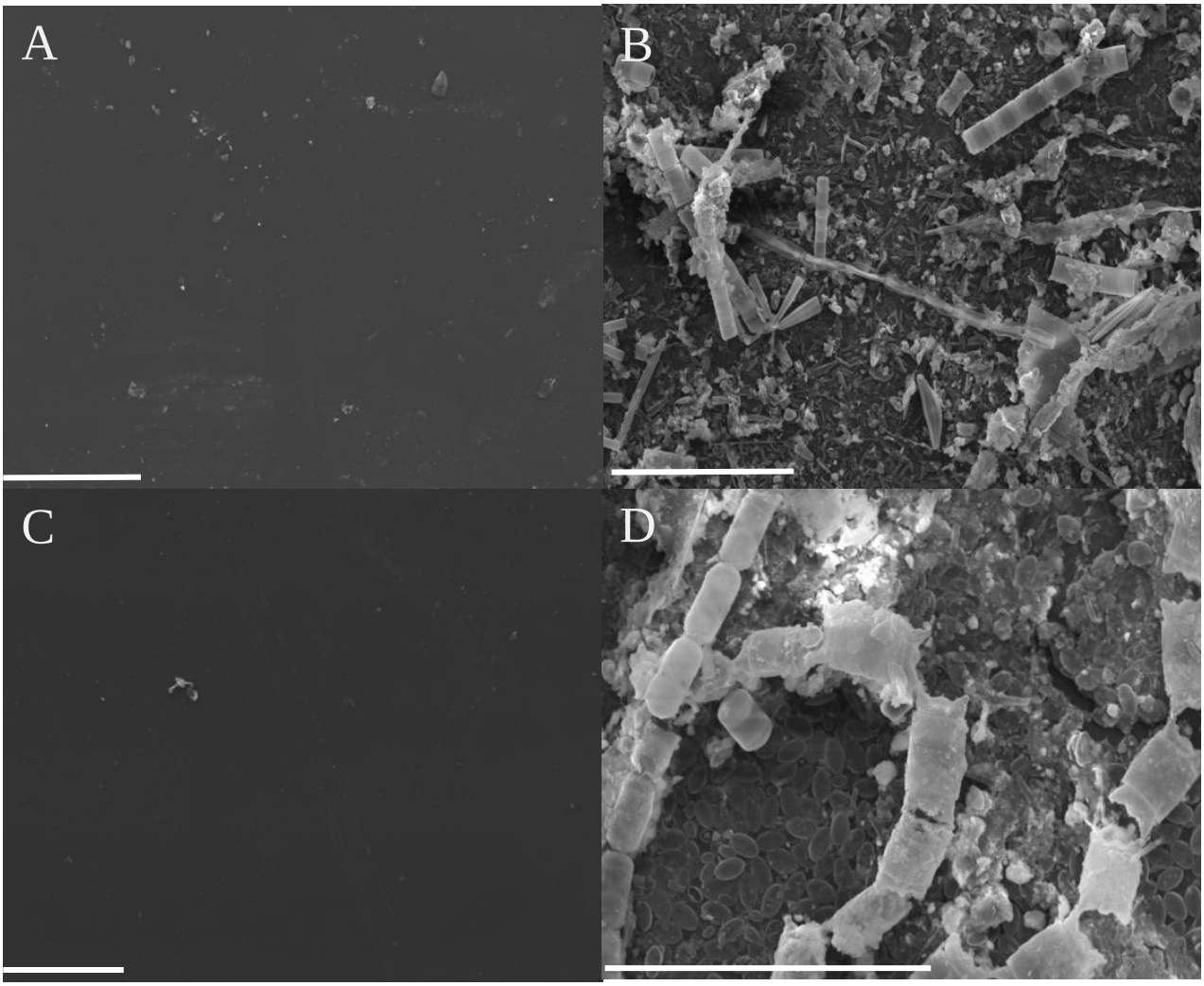


Figure 3.8 Scanning electron microscope (SEM) images of polymer surfaces and biofilm formation on polylactic acid (PLA) substrates from A) Dights Falls, Day 1 B) Dights Falls, Month 3 and C) Williamstown, Day 1 D) Williamstown, Month 3. Scale bars represent 250 μm.

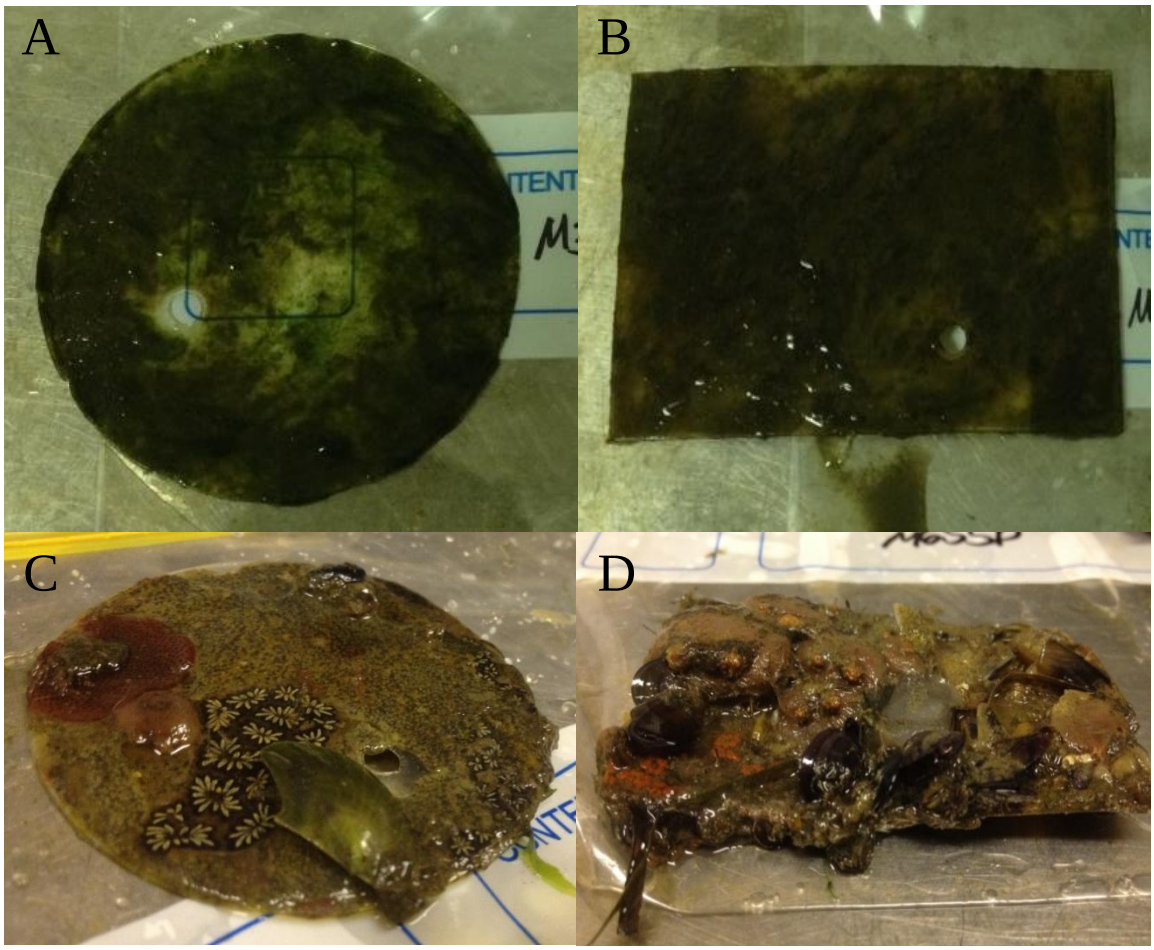


Figure 3.9Images of heavily biofouled plastic substrates. A) Polylactic acid (PLA) taken from Dights Falls at Month 3 B) Polypropylene (PP) taken from Dights Falls at Month 3 C) PLA taken from Williamstown at Month 6 D) PP taken from Williamstown at Month 6.

3.4 Discussion

This current studycompared variation in the structural properties of PP and PLA substrates, via analysis of surface hydrophobicity, tensile strength, crystallinity and chemical structureover a 12-month period in aquatic surface waters. There was no significant difference ($P>0.05$) when comparing the WCA or Young's Modulus between samples recovered on Day 1 or Month 12 for either the PP or PLA coupons. There was a significance difference ($P<0.05$) when comparing the Max Load between PLA samples recovered on Day 1 with samples at Month 12. However, this difference was due to an increase in the Max Load of the PLA over time. An increase in tensile strength is in contrast to what would be expected if the polymer was undergoing degradation through POD, as when chain scission of the polymer backbone occurs the chains are shortened leading to embrittlement (Singh and Sharma, 2008) and hence an expected decrease in Max Load and Youngs Modulus. Degradation via hydrolysis however, could potentially lead to the tensile strength increasing. Hydrolysis of PLA occurs more readily in the amorphous regions than crystalline regions, leading to the proportion of crystalline regions increasing and potentially the tensile strength of the polymer increasing (Yu *et al.*, 2012, Karamanlioglu *et al.*, 2017). The crystallinity of the PLA substrates increased between Day 1 and Month 6 (Crystallinity Day 1: 38.6 % and 40.0 % Crystallinity Month 6: 48.1 % and 50.0 %). However, only two sets of duplicate samples were taken for each sampling date and therefore the statistical significance of this apparent increase of crystallinity could not be determined. The FTIR spectra for samples from both Dights Falls and Williamstown for each respective coupon type (PP and PLA) were similar for samples taken on Day 1 and Month 6, indicating that chemical changes had not occurred for either polymer type. In addition, the Kruskal-Wallis pair-wise comparisons of the Young's Modulus of the PLAsubstrates did not vary significantly between different sampling dates; and similarly, a consistent trend over time in the variation of Max Load of the

PLA substrateswas not observed;as although the Max Load was significantly higher for the Month 12 samples when compared to the Day 1 and Month 9 samples, the Max Load for Month 12 samples was not significantly higher than for the Day 4, Month 3 or Month 6 samples. Therefore, the increase of Max Load between Day 1 PLA samples and Month 12 sample is likely stochastic. Similarly, no consistent trend appeared for the variation in the WCA of the PLA substrates. Furthermore, there were no significant differences in the Young's Modulus of the PP substrates between the different sampling dates; and there were no consistent trends in variation of the Max Load. Therefore, these data indicate that the bioplastic PLA is as structural stable as petroleum-based plastics (PP) in surface water environments for at least a period of 12 months, and potentially much longer. The reasons for why the Max Load of the PLA substrates increased for the Month 12 samples when compared to the Day 1 samples remain unknown.

Extensive biofouling of the polymer surfaces was also observed in this study (Figure 3.9) and a number a recent studies have similarly reported widespread colonisation of plastic surfaces in aquatic environments (Hoellein *et al.*, 2014, Reisser *et al.*, 2014, Oberbeckmann *et al.*, 2016). Similar to the results of this study, plastic substrates deployed in ocean surface waters were biofouled to such an extent that after 40 weeks, UV light penetration to the polymer surfaces was reduced by approximately 90% (O'Brine and Thompson, 2010). In contrast to the findings of this current study, microfragmentation of plastic substrates was observed after eight weeks of deployment, during an exposure study in an intertidal salt marsh at Charleston, SC, USA, where the plastic substrates were exposed to air for 6 h at low tide (Weinstein *et al.*, 2016). A factor leading to such elevated rates of deterioration could have been that the substrates were completely out of the water at low tide and had full sun exposure. This highlights the importance of UV radiation and POD for initial degradation of plastic via the breaking of the polymer chains, which may conversely, be reduced when plastics are in

aquatic environments (Hadad *et al.*, 2005, Shah *et al.*, 2008). In addition, wave action may be a significant process impacting plastic deterioration (Tsuji and Suzuyoshi, 2002) and is potentially the cause of sample loss during this current study. Although PLA films have previously been found to lose all tensile strength after being exposed in seawater for less than five weeks (Tsuji and Suzuyoshi, 2002), these films were much thinner than the PLA used in this current study, 25 μm - 50μm compared to 400μm, herein. Therefore, the PLA substrates used in this current study were likely more resistant to the physical effects of wave action.

During this current study the plastic coupons were prevented from sinking to the sediments. This may have limited the potential for biodegradation of the plastics since most microorganisms found to potentially be able to degrade plastics have been isolated from soil or sediment environments (See Section 1.5.2). Tosin *et al.* (2012) found that the biodegradation of Mater-Bi (a copolyester derived from vegetable oils and plant starches) using microcosms to simulate tidal zones and the pelagic domain, completely disintegrated within 9 months under simulated tidal conditions when buried under beach sand, while in contrast it took 2 years for the tensile strength to decrease by 66% under simulated pelagic conditions when kept afloat in a seawater aquarium.

In this current study, PLA was found to be as persistent in surface water environments as PP over a 12 month period with no significant changes in the WCA, Max Load,Young's Modulus or FTIR spectra for either plastic type, although there was some evidence that hydrolysis of the PLA substrates had occurred based on a decrease in the polymer crystallinity (that was unable to statistically tested for significance). It is hypothesised that significant degradation of the plastics was not observed because of the thick biofilms reducing the amount of UV light that was able to penetrate the polymer as well as being continually in water which would reduce the potential rate of thermal degradation of the polymers. Given the persistence of the PLA and PP coupons the next two chapters will

explore processes that occur on the plastic surfaces. These are; the accumumualtion of organic pollutants (Chapter 4) and the development of prokaryotic and eukaryotic microbial biofilm communities (Chapter 5).

Chapter 4: Accumulation of polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs) on polypropylene and polylactic acid substrates in aquatic environments

4.1 Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of brominated flame retardants (BFRs) that have been used in many consumer products such as in mattresses, furniture and electrical and electronic devices(Hale *et al.*, 2002). PBDEs have since become associated with disrupted thyroid homeostasis in humans (Linares *et al.*, 2015), potential developmental neurotoxicity (McDonald, 2005) and endocrine disruption (Costa *et al.*, 2008). The three main commercial formulations of PBDEs that were manufactured were "penta-BDEs", "octa-BDEs" and "deca-BDEs" (Alaee *et al.*, 2003) and were named to reflect the predominant congeners present in the mixtures with either five, eight or 10 degrees of bromination. Subsequent to penta-BDE, octa-BDE and deca-BDE PBDE formulations being listed as Persistent Organic Pollutants (POPs) under the Stockholm Convention(due to their environmental persistence, toxicity and propensity to bioaccumulate; Rahman *et al.* (2001), United Nations Environment Programme (2009)and United Nations Environment Programme (2017)) a market for the use of novel brominated flame retardants (NBFRs) to replace the use of PBDEs has since developed (Covaci *et al.*, 2011). To the best of my knowledge, quantification of NBFRs on plastic pollutants sourced from aquatic environments has not yet been undertaken;nor have there been any studies into the adsorption behaviour of either PBDEs or NBFRs onto bioplastics.

PBDEs have similar chemical structures compared to those ofpolychlorinated biphenyls (PCBs)consisting of two benzene rings with various degrees of bromination or chlorination, respectively (Figure 4.1); with trends of decreasing vapour pressures and water solubility and increasing octanol-water partitioning coefficients (K_{ow}) associated with increasing bromination

or chlorination (Table 4.1). The presence of PCBs on plastic recovered from marine environments has been reported in a number of studies(Ogata *et al.*, 2009, Rochman *et al.*, 2013a), with PCB concentrations ranging from tens to thousands of nanograms of PCB per gram (ng g^{-1}) of plastic. Fewer studies have investigated the presence and concentrations of PBDEs on plastics in marine environments;and havereportedPBDE concentrations of similar orders of magnitude as PCBs (See Section 1.7.1).

Chemical properties of NBFRs are comparable to PCBs and PBDEs, as NBFRs have low vapour pressures, low water solubilities and high octanol-water coefficients (Table 4.1).NBFRs have been detected on beached plastics, but are yet to be quantified from plastics obtained from marine environments (Rani *et al.*, 2015). Knowledge of the potential health risks from NBFRs exposure to human health is limited (Ezechiáš *et al.*, 2014). However, a recent review of the literature concluded there was a link between NBFR exposure and digestive system cancers and lymphoma in humans (Kim *et al.*, 2014). A group of NBFRs that were included in this current study consist of single or multiple benzene rings with varying degrees of bromination and/or functional groups (Figure 4.1); these NBFRs were pentabromotoluene (PBT), hexabromobenzene (HBB), pentabromoethylbenzene (PBEB), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB), 1,2-*bis*(2,4,6-tribromophenoxy) ethane (BTBPE) and decabromodiphenylethane (DBDPE). These six NBFRs were chosen for analysis because of their similar chemical characteristcs compared to PBDEs (Table 4.1) and these NBFRs had previously been detected in soils and house dust from around Melbourne, Australia(McGrath *et al.*, 2017b, McGrath *et al.*, 2018).

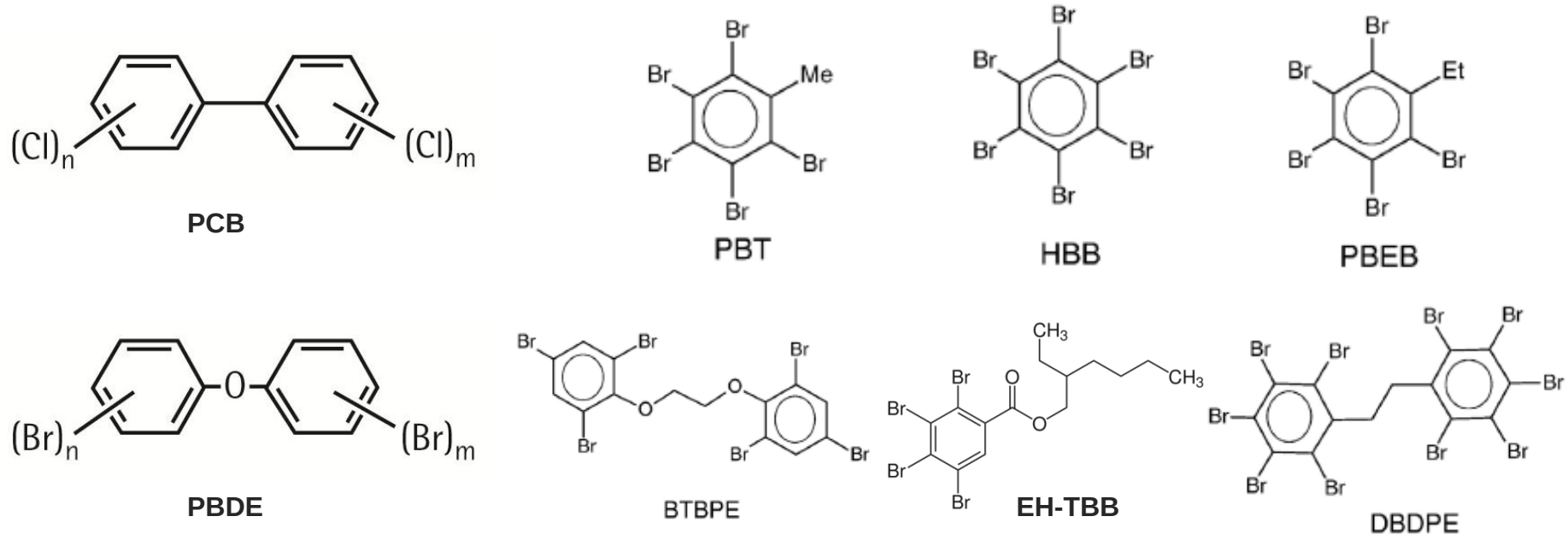


Figure 4.1 Chemical structures of polychlorinated biphenyls (PCBs),polybrominated biphenyl ethers (PBDEs) and selected novel brominated flame retardants (NBFRs) used in this current study.PBT = Pentabromotoluene, HBB = Hexabromobenzene, PBEB = Pentabromoethylbenzene, BTBPE = 1,2-*bis*(2,4,6-tribromophenoxy) ethane,EH-TBB = 2-ethylhexyl-2,3,4,5-tetrabromobenzoate, DBDPE = Decabromodiphenylethane.Adapted from Sightline Institute (2018).

Table 4.1 Physico-chemical properties of polychlorinated biphenyls (PCBs),polybrominated biphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs).

Compound	Congener	Number of chlorines or bromines	Molecular weight (g mol^{-1})	Vapour pressure (Pa) (25°C)	Water solubility (g L^{-1}) (25°C)	Octanol-water coefficient (log K_{ow})
PCB	**PCB-105**	5	326.4	8.7×10^{-4} [a]	3.4×10^{-6} [a]	6.65 [a]
	PCB-153	6	360.9	5.0×10^{-7} [a]	9.0×10^{-7} [a]	6.69 [a]
	PCB-180	7	395.3	1.3×10^{-6} [a]	2.0×10^{-7} [a]	7.36 [a]
PBDE	**BDE- 28**	3	406.9	2.19×10^{-3} [b]	1.7×10^{-4} [b]	5.94 ± 0.15 [b]
	BDE- 47	4	485.8	2.53×10^{-4} [b]	5.4×10^{-5} [b]	6.81 ± 0.08 [b]
	BDE- 99	5	564.6	4.29×10^{-5} [b]	4.35×10^{-5} [b]	7.32 ± 0.14 [b]
	BDE- 100	5	564.6	2.86×10^{-5} [b]	4.80×10^{-5} [b]	7.24 ± 0.16 [b]
	BDE- 153	6	643.6	5.26×10^{-6} [b]	2.0×10^{-5} [b]	7.90 ± 0.14 [b]
	BDE- 154	6	643.6	3.8×10^{-6} [b]	1.10×10^{-5} [b]	7.82 ± 0.16 [b]
	BDE- 183	7	722.5	4.68×10^{-7} [b]	1.5×10^{-6} [c]	8.27 ± 0.26 [b]
	BDE- 209	10	959.2	3.2×10^{-9} [c]	1.92×10^{-6} [a]	9.1 [c]
NBFR	**PBT**	5	486.6	1.22×10^{-3} [d]	7.80×10^{-4} [d]	5.87 ± 0.62 [d]
	HBB	6	551.5	2.1×10^{-6} [d]	3.0×10^{-6} [d]	6.07 [d]
	PBEB	5	500.7	3.2×10^{-4} [d]	3.50×10^{-4} [d]	6.40 ± 0.62 [d]
	BTBPE	6	687.6	3.9×10^{-10} [d]	1.9×10^{-5} [d]	8.99[e]
	EH-TBB	4	549.9	3.7×10^{-7}[d]	1.1×10^{-8} [d]	7.73 [d]
	DBDPE	10	971.2	6.0×10^{-15} [d]	2.10×10^{-7} [d]	11.1 [d]

[a]International Agency for Research on Cancer (IARC) (2016), [b]Yue and Li (2013), [c]Cousins *et al.* (2014), [d]Covaci *et al.* (2011)[e]Kuramochi *et al.* (2014). PBT = Pentabromotoluene, HBB = Hexabromobenzene, PBEB = Pentabromoethylbenzene, BTBPE = 1,2-*bis*(2,4,6-tribromophenoxy) ethane, EH-TBB = 2-ethylhexyl-2,3,4,5-tetrabromobenzoate, DBDPE = Decabromodiphenylethane.

Brominated flame retardants such as PBDEs and NBFRs are transported in the environment by multiple pathways including via movement of contaminated sediment in rivers (Herrero *et al.*,

2018), or global transport by being bound to airborne particulate matter (de la Torre *et al.*, 2018). The prevalence of PBDEs has been widely covered in reviews studying humans (Darnerud *et al.*, 2001, Covaci *et al.*, 2011, Fromme *et al.*, 2016), biota (Law *et al.*, 2003) and natural-(Katima *et al.*, 2017, McGrath *et al.*, 2017a) and built- environments (Besis and Samara, 2012) (Table 4.2). Additionally, high rates of PBDE and NBFR release have been associated with e-waste (electronic waste such as circuit boards) processing (McGrath *et al.*, 2017a) and sewage sludge (Clarke *et al.*, 2008).

PBDEs and NBFRs have been identified in remote Arctic apex predators such as Polar bears (*Ursus maritimus*) and Peregrine falcons (*Falco peregrinus*). Concentrations of PBDEs in adipose tissue samples (collected between 1994 to 2002) from Polar bears have ranged between 27-114 ng g^{-1} lw (Muir *et al.*, 2006); while concentrations of theNBFR, 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), have been detected at concentrations of no more than three orders of magnitude lower than concentrations of PBDEs in Polar bear adipose tissue (collected in 2012)(Vorkamp *et al.*, 2015). The concentrationof PBDEs in lipid samples (collected between 1987-1999) from Peregrine falcons eggs have ranged between 2200-2700 ng g^{-1} lw (Lindberg *et al.*, 2004). In urban environments, PBDE in lipid samples (collected between 1986-2007) from Peregrine falcons eggs have ranged between 0.08–53.1 ng g^{-1} lw (Park *et al.*, 2009).The high concentration of PBDEs from the eggs from inland Peregrine falcon may be due to the falcons' diet being reliant on Rock pigeons (*Columba livia*) which are known to consume waste material (Park *et al.*, 2011).

One of the characteristics of PBDEs that lead to them being defined as POPs is their ability to bioaccumulate (United Nations Environment Programme, 2009). PBDEs begin to bioaccumulate in humans before we reach cradle, and only stop accumulating once we reach the grave (Siddiqi*et al.*, 2003). A study investigating PBDEs in human umbilical cord plasma (43 samples from women in Denmark), detected PBDEs in all samples, the median concentration was 958 pg

g^{-1} lw(Frederiksen *et al.*, 2010).Concerns about PBDE exposure to infants via human breast milk were first raised by Meironyté *et al.* (1999), who determined that total concentrations of eight PBDE congeners (excluding BDE-209) in breast milk from Swedish women ranged between 0.07-4.02 ng g^{-1} lw. By comparison a more recent study of PBDEs in human breast milk from Tanzanian women found the total concentration of seven PBDE congeners(excluding BDE-209) were up to two orders of magnitude higher and ranged between <LOD- 785 ng g^{-1} lw (Müller *et al.*, 2016). The relatively high concentrations of PBDEs in breast milk from Tanzanian women may be associated with their weekly consumption of fish and a clay based mineral supplement that pregnant woman often consume (Müller *et al.*, 2016).A pair of studies from Japan investigating concentrations of PBDEs (Kunisue *et al.*, 2007)andhexabromocyclododecane (HBCD) (Isobe *et al.*, 2009) from predominately middle-aged human adipose tissue(collected between 2003–2004, average age 65.5 ± 3 years)and found that PBDE and HBCDconcentrations rangedbetween1.8–46 ng g^{-1} lw and 0.85–39 ng g^{-1} lw, respectively. Males were found to have on average; twice the concentration of PBDEs and HBCDs as females; the authors postulated that this was potentially because of the loss of brominated flame retardants (BFRs) that women experience through breast feeding(Kunisue *et al.*, 2007, Isobe *et al.*, 2009).

Table 4.2 Examples of PBDEs and NBFRs concentrations in various human, biota, natural environments and built environments.

Analytes	Concentration of analytes (Range)	Sample type	Location	Reference
		Biota		
HBCD	150- 250 ng g^{-1} lw	Peregrine falcons (*Falco peregrinus*) eggs	Sweden	(Lindberg *et al.*, 2004)
PBDE (∑8 congeners)	2200-2700 ng g^{-1} lw	Peregrine falcons (*Falco peregrinus*) eggs	Sweden	(Lindberg *et al.*, 2004)
PBDE (∑8 congeners*)	2.65-9.72 ng g^{-1} wet wt	Polar bear (*Ursus maritimus*) blood plasma	Svalbard	(Verreault *et al.*, 2005)
PBDE (∑8 congeners*)	8.23-67.5 ng g^{-1} wet wt	Glaucous gulls (*Larus hyperboreus*)	Svalbard	(Verreault *et al.*, 2005)
BTBPE	0.11 ng g^{-1} lw	Northern fulmars (*Fulmarus glacialis*) eggs	United Kingdom	(Karlsson *et al.*, 2006)
PBDE (∑8 congeners)	3.14 ng g^{-1} lw	Northern fulmars (*Fulmarus glacialis*) eggs	United Kingdom	(Karlsson *et al.*, 2006)
PBDE (∑4 congeners)	27.0-114 ng g^{-1} lw	Polar bear (*Ursus maritimus*) adipose tissue	Svalbard	(Muir *et al.*, 2006)
PBDE (∑15 congeners)	0.08–53.1 ng g^{-1} lw	Peregrine falcons (*Falco peregrinus*) eggs	California, USA	(Park *et al.*, 2009)
NBFR (∑3 compounds)	0.48 ng g^{-1} ww	Polar bear (*Ursus maritimus*) adipose tissue	Greenland	(Vorkamp *et al.*, 2015)
		Human		
PBDE (∑8 congeners[#])	0.07-4.02 ng g^{-1} lw	Breast milk	Sweden	(Meironyté *et al.*, 1999)
BDE-209	<LOD- 17.4 ng g^{-1} lw	Blood serum	Sweden	(Karlsson *et al.*, 2007)
DBDPE and BTBPE	<LOD	Blood serum	Sweden	(Karlsson *et al.*, 2007)
PBDE (∑13 congeners)	1.8–46 ng g^{-1} lw	Adipose tissue	Japan	(Kunisue *et al.*, 2007)
HBCD	0.85–39 ng g^{-1} lw	Adipose tissue	Japan	(Isobe *et al.*, 2009)

PBDE (∑21 congeners)	0.41-1980 ng g^{-1} lw	Blood serum	China	(Zhu *et al.*, 2009)
PBDE (∑7 congeners[#])	<LOD- 785 ng g^{-1} lw	Breast milk	Tanzania	(Müller *et al.*, 2016)
HBCD	<LOD-28.1 ng g^{-1} lw	Breast milk	Tanzania	(Müller *et al.*, 2016)
Natural environment				
PBDE (∑ 7 congeners[#])	<0.02-18 ng g^{-1} dw	Estuarine sediment	Belgium	(Voorspoels *et al.*, 2004)
PBDE (∑8 congeners)	88-812 µg kg^{-1}	River sediment	Spain	(Cristale *et al.*, 2013a)
BDE-209	17 -295 ng L^{-1}	River Aire water	United Kingdom	(Cristale *et al.*, 2013b)
NBFR (∑2 compounds)	0.16-0.40 ng L^{-1}	River Aire water	United Kingdom	(Cristale *et al.*, 2013b)
PBDE (∑8 congeners)	<LOD-13,200 ng g^{-1} dw	Melbourne Soil	Australia	(McGrath *et al.*, 2016)
NBFR (∑6 compounds)	<LOD-385 ng g^{-1} dw	Melbourne Soil	Australia	(McGrath *et al.*, 2017b)
Built environment				
NBFR (∑4 compounds)	14-39 ng g^{-1} dust	Indoor house dust	New Zealand	(Ali *et al.*, 2012)
PBDE (∑8 congeners)	120-1700,000 ng g^{-1}	Melbourne house dust	Australia	(McGrath *et al.*, 2018)
NBFR (∑7 compounds)	1.1- 10,000 ng g^{-1}	Melbourne house dust	Australia	(McGrath *et al.*, 2018)

PBDE= Polybrominated diphenyl ethers; NBFR= Novel brominated flame retardant; DBDPE = Decabromodiphenylethane;BTBPE = 1,2-*bis*(2,4,6-tribromophenoxy)ethane;HBCD = Hexabromocyclododecane;ww = wet weight; dw = dry weight; lw = lipid weight

*methoxylated (MeO) and hydroxylated (OH) PBDEs

[#]excluding BDE-209.

Only two previous studies have investigated PBDEson plastic debris in marine environments, studying plasticsrecovered from the Central and North Pacific Gyres (Hirai et al., 2011, Chen et al., 2018). PBDEs quantified had concentrations ranging between 0.02-9,900 ng g^{-1} (with a mean of 261 ng g^{-1}± 224 ng g^{-1})(Hirai *et al.*, 2011)and 0.6ng g^{-1}-188 ng g^{-1} (with a mean of 20 ng g^{-1}±5 ng g^{-1})(Chen *et al.*, 2018). The PBDE concentration in the Hirai *et al.* (2011) study had a large standard error of the mean because one PP plastic fragment had a comparatively extremely high concentration (9,909 ng g^{-1}). The median PBDE concentrations from both studies were similar; with concentrations of 3.65 ng g^{-1}(Hirai *et al.*, 2011) and 6.1 ng g^{-1}(Chen *et al.*, 2018). Similarly, the mean hexabromocyclododecane (HBCD) concentration was 27 ngg^{-1}± 18 ng g^{-1}, while the median concentration was only 0.9 ng g^{-1}(Chen *et al.*, 2018).These data indicate that there is large heterogeneity of in the concentrations of PBDE between different plastic samples collected from the same locations.

Current global bioplastic production capacity is estimated to be 2.05 million tonnes per annum, and is expected to increase to 2.44 million tonnes per annum by 2022 (European Bioplastics, 2017). Furthermore, the production of NBFRs is estimated to be approximately 100,000 metric tonnes per year (Harju *et al.*, 2009). As the previous chapter of this thesis demonstrated, bioplastics such as polylactic acid (PLA) andpetroleum-based plastics such as polypropylene (PP) are highly recalcitrant in aquatic environments. Therefore, there is a need to understand if there is potential for bioplastics to adsorb PBDEs and NBFRs in natural aquatic environments in a similar manner as can occur on petroleum-based plastics; which are known to facilitate POP transport in aquatic environments (Teuten *et al.*, 2007).The aim of this current study was to determine the potential for brominated flame retardants (PBDEs and NBFRs) to adsorb and accumulate on PP and PLA with comparison to glass substrates, in an exposure experiment in an urban estuarine system over a 12-month period. To achieve this aim, a novel analytic method to

process the PLA substrateswas successfully developed and utilised whereby the coupons were subject to extraction via a series of sonication and vortexing steps in an organic solvent (1 :4, dichloromethane: hexane). It was expected that the concentrations of the absorbed PBDEs and NBFRson PP, PLA and glass substrates would increase over the 12-month experiment, similar to the results observed for the adsorption of PCBs onto PP and PE byRochman *et al.* (2013a). This is the first study to investigate the potential for NBFRs and POPs such as PBDEs to adsorb onto PLA in surface watersunder natural conditions.

4.2 Methods

4.2.1 Chemical standards and materials

Isotopically labelled chemical standards were purchased from Wellington Laboratories (Guelf, ONT, Canada). Non-isotopically labelled native standards were purchased from AccuStandard Inc. (New Haven, CT, USA) (Table 4.3).

All solvents used in extraction, clean up and analysis were of pesticide grade(purity minimum 99.8 %), unless otherwise stated. *Iso*-octane, *n*-hexane and dichloromethane (DCM) were obtained from Honeywell Burdick & Jackson (Muskegon, MI, USA). Florisil (60–100 mesh $MgSiO_3$), alumina (Al_2O_3) and anhydrous sodium sulphate (Na_2SO_4) were from Sigma Aldrich (St Louis, MO, USA), Davisil silica (200–425 mesh amorphous SiO_2) from Grace Davison Discovery Science (Rowville, VIC, Australia) and Hydromatrix diatomaceous earth from Varian Inc. (Santa Clara, CAL, USA).

Table 4.3 Analytical chemical standards used in this study.

Compound	Abbreviation	Category[a]	Manufacturer
2,3,4,5,6-Pentabromotoluene	PBT	Native	AccuStandard
2,3,4,5,6-Pentabromoethylbenzene	PBEB	Native	AccuStandard
Hexabromobenzene	HBB	Native	AccuStandard
2-Ethylhexyl-2,3,4,5-tetrabromobenzoate	EH-TBB	Native	AccuStandard
1,2-Bis(2,4,6-tribromophenoxy)ethane	BTBPE	Native	AccuStandard
Decabromodiphenylethane	DBDPE	Native	AccuStandard
2,4,4'-Tribromodiphenyl ether	BDE-28	Native	AccuStandard
2,2',4,4'-Tetrabromodiphenyl Ether	BDE-47	Native	AccuStandard
2,2',4,4',5-Pentabromodiphenyl ether	BDE-99	Native	AccuStandard
2,2',4,4',6-Pentabromodiphenyl ether	BDE-100	Native	AccuStandard
2,2',4,4',5,5'-Hexabromodiphenyl ether	BDE-153	Native	AccuStandard
2,2',4,4',5,6'-Hexabromodiphenyl ether	BDE-154	Native	AccuStandard
2,2',3,4,4',5',6-Heptabromodiphenyl ether	BDE-183	Native	AccuStandard
Decabromo diphenyl ether	BDE-209	Native	AccuStandard
2,2′,4,4′-Tetrabromo[$^{13}C_{12}$]diphenyl ether	$^{13}C_{12}$-BDE-47	Surrogate IS	Wellington
2,2′,4,4′,5-Pentabromo[$^{13}C_{12}$]diphenyl ether	$^{13}C_{12}$-BDE-99	Surrogate IS	Wellington
2,2′,4,4′,5,5′-Hexabromo[$^{13}C_{12}$]diphenyl ether	$^{13}C_{12}$-BDE-153	Surrogate IS	Wellington
Decabromo[$^{13}C_{12}$]diphenyl ether	$^{13}C_{12}$-BDE-209	Surrogate IS	Wellington
3,4,4′-Tribromodiphenyl ether	BDE-37	Recovery IS	AccuStandard
3,3′,4,4′-Tetrabromodiphenyl ether	BDE-77	Recovery IS	AccuStandard

Adapted from (McGrath *et al.*, 2017b). [a]Natives = non-$^{13}C_{12}$- mass labelled standards, Surrogate IS= $^{13}C_{12}$- masslabelled internal standards, Recovery IS = non-$^{13}C_{12}$- mass labelled recovery standards.

4.2.2 Selective Pressurised Liquid Extraction (S-PLE)

A Dionex ASE 200 Accelerated Solvent Extraction (ASE) system (Dionex, Sunnyvale, USA) was used for the extraction of PBDEs and NBFRs from the PP and glass samples. Only the PP and glass samples were processed using the ASE, as the PLA samples melted using this

technique (data not shown). The conditions of the ASE were: temperature: 40 ^{o}C, pressure: 1500 psi, solvents: 1:4 dichloromethane: hexane, flush volume: 60 %, static time: 5 min, static cycles: 3, N_2 purge time: 2 min. A selective pressurised liquid extraction (S-PLE) was applied to PP and glass samples and the 33 mL ASE cells (metal canisters used to contain the sample and sorbents in the S-PLE process) were packed with sorbents in the order from bottom to top, with the sorbents: 3 g of Florisil, 3 g of alumina, 3 g of silica powder and 3 g sodium sulphate. After the samples were then added to the ASE cells, Hydromartix was added to fill the ASE cells to within 2 cm from the top.

4.2.3 Dual vortex and sonication extraction method of PBDEs and NBFRs from PLAsubstrates

A novel PBDE and NBFR extraction method was required for the PLA substrates because the S-PLE method used for the PP and glass substrates resulted in the PLA coupons melting, and forming a discoloured extract. Several unsuccessful methods were attempted before a successful method was developed (see Section 4.3.1). These methods were: a method whereby samples were wiped several times with acetone soaked antiseptic wipes and a liquid-liquid extraction method using concentrated sulphuric acid and hexane.

The successful PBDEs and NBFR extraction method from the PLA samples involved using a dual vortex and sonication extraction process. PLA samples were placed into a 60 mL glass ASE vial, and 30 mL of organic solvent, (1:4 DCM: Hexane) was added. The ASE vials were vortexed using a Heidolph shaker (John Morris, Melbourne, Australia) for 15 min, then placed in an ultrasonic water bath (Unisonics, New York, USA) and sonicated for 15 min. Extracts were transferred to clean glass ASE vials via Pasteur pipettes. This process of vortexing and sonicating the samples in solvent was further repeated twice.The pooled extractwas then evaporated under gentle N_2 stream using a Biotage ® Turbovap ® LV evaporator (Biotage,

Uppsala, Sweden) until approximately 5 mL of solvent remained. Extracts were then filtered through pre-packed Pasteur pipettes into clean glass ASE vials. The Pasteur pipettes were loaded (from bottom to top) with glass wool, 2 g of Florisil, 2 g of alumina and covered with a small amount of glass wool. Pasteur pipettes were flushed twice with 100% DCM to rinse through the analytes.

4.2.4 Combined method for transferring polypropylene, polylactic acid and glass extracts into gas chromatrography vials

All PP, PLA and glass extracts were then evaporated until approximately 2 mL of solvent remained using a Biotage ® Turbovap ® LV evaporator (Biotage, Uppsala, Sweden) under a gentle N_2 stream. The sides of the vial were rinsed several times with DCM and evaporated. Theresulting solutions were transferred into clean 2 mL amber gas chromatography (GC) vials and evaporated to dryness. Each extract was then reconstituted in a 100 µL solution of the recovery standards [BDE-37 and BDE-77 (1000 ng mL^{-1})] in isooctane.

4.2.5 Analysis and quantification of PBDEs and NBFRs

An Agilent 7000C gas chromatograph (Agilent, Santa Clara, U.S.A) coupled to a triple quadrupole mass spectrometer (GC-MS/MS) operated in electron ionisation (EI) mode was used for PBDE and NBFR analysis (DB-5MS column; 15 m x 0.18 mm internal diameter, 0.18 µm film thickness) using a method previously developed by McGrath *et al.* (2017b). Briefly,the injection volume was 2 µL, the initial inlet temperature was 100 ^{o}C for 0.2 min. The inlet temperature was then ramped at 900 ^{o}C min^{-1} to 330 ^{o}C. The initial oven temperature was 80 ^{o}C for 1 min, it was then ramped to 230 ^{o}C at a rate of 37.5 ^{o}C min^{-1}. The rate was then decreased to 30 ^{o}C min^{-1} until the oven reached a temperature of 325 ^{o}C. Ultrapure helium was used as a carrier gas. The initial helium flow rate was 1.8 mL min^{-1} for 8.25 min, the flow rate was then

increased to 4 mL min^{-1} at a rate of 100 mL min^{-1}. The total run time was 13.5 min. Target PBDEs and NBFRs analytes were determined by retention time and two transition ions using Agilent MassHunter quantitative analysis software (v. B.06.00) (Table 4.4). Analytes were quantified against a five point calibration curve. Parameters to qualify analytes as being detected were the signal to noise ratio (S/N) in the quantitative ion transition being of at least three and the GC retention time being within ±5% of those of the analytical calibration standards. Parameters to qualify analytes as being quantifiable were that the S/N ratio exceeded 10 in the quantitation transition ion, and wasalso at least three in the qualitative transition and also that the ratio between the two monitored transitions was within ±20% of those measured in calibration standards.

Table 4.4 GC-(EI)-MS/MS acquisition parameters and quantitation parameters for PBDEs and NBFRs.

		Quantitation Transition			Confirmation Transition		
Compound	**Retention Time (RT) (min)**	**T1 (m/z)**	**Dwell (ms)**	**CE (eV)**	**T2 (m/z)**	**Dwell (ms)**	**CE (eV)**
BDE-28	5.695	405.8→246.0	10	20	408.0→248.1	10	5
PBT	5.76	485.5→247.0	10	20	485.5→326.0	10	20
BDE-37	5.88	405.8→246.0	10	20	408.0→248.1	10	5
PBEB	5.89	499.7→484.6	10	20	499.7→420.5	10	20
HBB	6.26	552.0→391.8	10	25	549.5→389.7	15	25
^{13}C-BDE-47	6.39	497.7→338.0	12	25	495.7→336.1	12	45
BDE-47	6.39	486.0→326.0	10	45	325.8→138.0	10	20
BDE-77	6.65	486.0→326.0	10	45	326.0→138.0	10	20
BDE-100	6.82	563.6→403.7	10	35	403.7→296.7	16	35
^{13}C-BDE-99	7.02	577.7→417.8	10	40	417.3→309.0	13	55
BDE-99	7.02	563.6→403.7	10	20	565.6→405.6	10	20
EH-TBB	7.02	420.5→233.0	10	30	420.5→311.5	10	30
BDE-154	7.357	643.6→483.8	10	25	483.7→374.9	14	25
BDE-153	7.567	643.6→483.8	10	40	483.7→323.6	10	40
^{13}C-BDE-153	7.62	6.55→495.8	10	25	495.7→386.9	10	25
BDE-183	8.121	721.6→561.8	21	20	561.7→454.9	10	25
BTBPE	8.34	356.5→118.0	16	40	356.5→90.0	16	60
^{13}C-BDE-209	11.56	811.8→651.4	61	55	809.7→649.5	57	55
BDE-209	11.56	799.4→639.5	67	55	797.7→637.7	52	55
DBDPE	12.96	484.5→324.5	113	25	484.5→403.5	136	55

T1= First transition ion, T2 = Second transition ion, CE = Collision energy, BDE-= Polybrominated diphenyl ether congener, PBT= Pentabromotoluene, PBEB = Pentabromoethylbenzene, HBB= Hexabromobenzene, EH-TBB= 2-ethylhexyl-2,3,4,5-tetrabromobenzoate,BTBPE= 1,2-*bis*(2,4,6-tribromophenoxy) ethane, DBDPE = Decabromodiphenylethane.

4.2.6 Quality assurance and quality control (QA/QC)

A series of three method quality assurance and quality controls (QA/QC) were analysed with every extraction batch. These consisted of a method blank, laboratory control sample (LCS) and matrix spike. The method blanks involved extracting and processing following the same protocols for the S-PLE and dual vortex/ sonication extraction methods without any sample being present. Trace level contamination was observed in method blanks for all compounds except BDE-28, PBT, EH-TBB, DBDPE and PBEB. For analytes with detectable contamination, method detection limits (MDLs) and method quantitation limits (MQLs) were set to meet 95% and 99% confidence intervals, respectively, of the mean contamination levels within method blanks. The MDLs and MQLs for the analytes that did not have detectable contamination were set at 95% and 99% of the lowest calibration standard (1 ng g^{-1}). The instrument detection limits (IDLs) were determined from the lowest analyte detected that had a S/N ratio of 3 for the response of the quantitative transition ion. The instrument quantitation limits (IQLs) were determined from the lowest analyte detected that had S/N ratios of 10 in the response of quantitation ion and an S/N ratio of 3 for the confirmation transition ion. LCS and matrix spikes were prepared by spiking either ASE cells pre-packed with sorbents for the S-PLE extraction method (for PP and glass samples), or an empty ASE vial for the vortex-sonication method (for the PLA samples) with a known amount of each target analyte with no sample (LCS) or with either PP or PLA (matrix spikes). The LCSs and matrix spikes were spiked with non-$^{13}C_{12}$- mass labelled native PBDE and NBFR standards: NBFRs, PBT, PBEB, HBB (10 ng), EH-TBB and BTBPE (20 ng) and DBDPE (200 ng); PBDEs, BDE- 28, BDE- 47, BDE- 99, BDE- 100, BDE- 153, BDE- 154, BDE- 183 (10 ng) and BDE-209 (100 ng). The LCS were extracted and processed following the same protocols as for the S-PLE and dual vortex and sonication extraction methods. The matrix spikes with PP samples were processed using the S-PLE method

(See 4.2.2), while matrix spikes with PLA samples were processed using the dual vortex/sonication method (See 4.2.3).

4.2.7 Data analysis

Statistical comparisons between the adsorption of pollutants onto different substrate types between the five different sample locations could not be made due to the small sample sizes resulting from samples being lost over the course of the exposure experiment especially after Month 3 and additionally as different extraction methods were used for the PP and glass substrates compared to the PLA substrates. Consequently,changes in the frequency of detection of absorbed pollutants and of the concentrations of the PBDEs and NBFRs absorbing onto substrates over the 12-month exposure experiment are discussed across all substrates and sites.

4.3 Results

4.3.1 Method development for extracting PBDEs and NBFRs from PLA

The S-PLE extraction method of the PLA samples (See 4.2.2) resulted in the extract being discoloured (Figure 4.2A). Therefore, a novel extraction method was developed (see Section 4.2.3). The initial method development, attempt whereby acetone soaked antiseptic wipes were rubbed on the PLA substrates, resulted with the antiseptic wipes also melting and left fibre fragments in the ASE apparatus (Figure 4.2B). Following this, a liquid-liquid extraction method using a combination of concentrated sulphuric acid and hexane was attempted using a separating glass funnel. However, this method was abandoned due to the excessive volumes of concentrated sulphuric acid and hexane required as well as health and safety considerations. An extraction method using a combination of vortexing and sonication was successfully developed resulting in a clear extract (Figure 4.2C and Figure 4.2D).

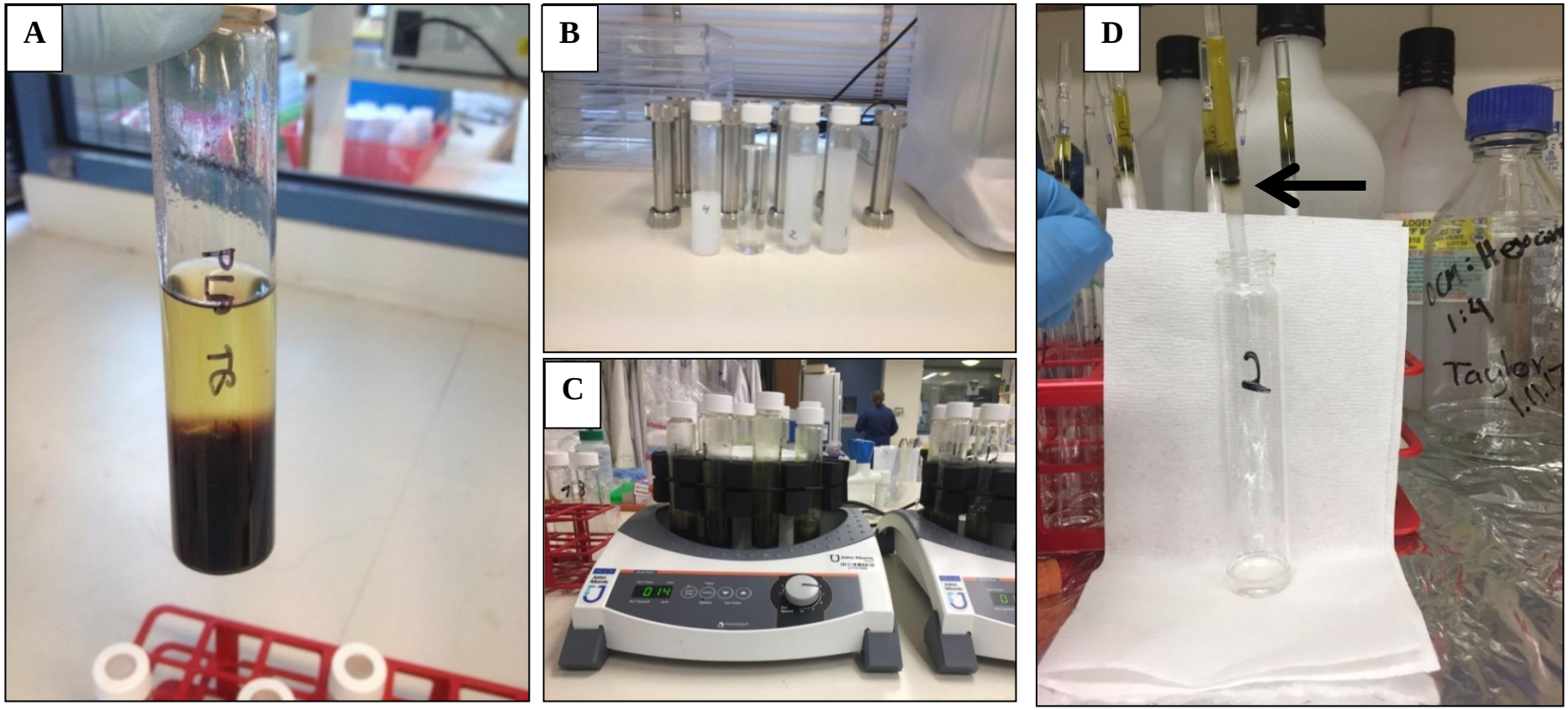


Figure 4.2Extraction and clean-up method development of polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs) from polylactic acid (PLA)A) initial extract using the selective pressurised liquid extraction (S-PLE) method resulting in a burnt, discoloured extract B) S-PLE method using antiseptic wipes, resulting in cloudy extracts C) vortexing PLA samples D) filtering extracts (after vortexing and sonicating) through Pasteur pipettes, resulting in a clear extract. Note, the pigments being trapped by the sorbents (arrowed).

4.3.2 Analytical method validation

Analyte recoveries for the S-PLE and dual vortex/sonication extraction methods of the matrix spikes were validated for the PBDEs and NBFRs (Table 4.5). For the S-PLE and dual vortex/sonication methods, the recoveries for all of the PBDE congener recoveries were of at least 75% (BDE-28 75% ± 11% and BDE-183 77% ± 12% respectively). EH-TBB had the lowest recoveries for both S-PLE and dual vortex/sonication methods, (18% ± 6% and 61% ± 20%, respectively), and therefore was removed from further analysis. The MDLs and MQLs are presented in Table 4.6. The MDLs were calculated with confidence intervals of 95%, while the MQLs were calculated with confidence intervals of 99%. The GC-MS analytical method failed to detect the spiked BTBPEs, and this compound was therefore removed from further analysis.

Table 4.5 PBDE and NBFR recoveries for both the S-PLE (n = 8) and dual vortex/ sonication (n = 7) extraction methods.

	Recovery (%)	
Compound	**S-PLE method**	**Dual vortex/ sonication method**
BDE- 28	75 ± 11	89 ± 2
BDE- 47	78 ± 7	100 ± 3
BDE- 99	101 ± 6	91 ± 2
BDE- 100	100 ± 14	92 ± 1
BDE- 153	89 ± 8	98 ± 1
BDE- 154	83 ± 7	100 ± 1
BDE- 183	79 ± 11	77 ± 12
BDE- 209	133 ± 27	107 ± 4
PBT	82 ± 8	74 ± 3
HBB	91 ± 6	75 ± 4
PBEB	45 ± 11	78 ± 2
EH-TBB	18 ± 6	61 ± 20
DBDPE	129 ± 16	85 ± 3

PBDE= Polybrominared diphenyl ethers, NBFR = Novel brominated flame retardants, BDE-= Polybrominated diphenyl ether congener, PBT= Pentabromotoluene, HBB= Hexabromobenzene, PBEB= Pentabromoethylbenzene, EH-TBB= 2-ethylhexyl-2,3,4,5-tetrabromobenzoate,DBDPE= Decabromodiphenylethane.

Table 4.6 Instrument and analytical detection and quantitation limits of the glass, PP and PLA substrates.

	IDL	IQL	MDL (ng g^{-1})			MQL (ng g^{-1})		
Compound	**(ng mL^{-1})**	**(ng mL^{-1})**	**Glass**	**PP**	**PLA**	**Glass**	**PP**	**PLA**
BDE- 28	1.0	1.0	0.0001	0.07	0.003	1.0	1.0	1.0
BDE- 47	2.1	3.1	0.0003	0.13	0.01	0.06	0.19	0.29
BDE- 99	0.3	0.4	0.0000	0.02	0.001	0.01	0.02	0.04
BDE- 100	2.2	2.8	0.0003	0.14	0.01	0.06	0.17	0.26
BDE- 153	1.6	1.9	0.0002	0.10	0.01	0.04	0.11	0.18
BDE- 154	1.7	2.1	0.0002	0.11	0.01	0.04	0.13	0.20
BDE- 183	5.9	8.4	0.0008	0.38	0.02	0.17	0.51	0.79
BDE- 209	280	452	0.04	18	0.97	9.3	27	42
PBT	1.0	1	0.0001	0.07	0.003	1.0	1.0	1.0
HBB	3.3	5.0	0.0005	0.22	0.01	0.10	0.30	0.47
PBEB	1.0	1.0	0.0001	0.07	0.003	1.0	1.0	1.0
EH-TBB	1.0	1.0	0.0001	0.07	0.003	1.0	1.0	1.0
BTBPE	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
DBDPE	1.0	1.0	0.0001	0.07	0.003	1.0	1.0	1.0

PP= Polypropylene, PLA= Polylactic acid, IDL= Instrument detection limit, IQL= Instrument quantitation limit, MDL= Method detection limit, MQL = Method quantitation limits, BDE-= Polybrominated diphenyl ether congener, PBT= Pentabromotoluene, HBB = Hexabromobenzene, PBEB= Pentabromoethylbenzene, EH-TBB= 2-ethylhexyl-2,3,4,5-tetrabromobenzoate,BTBPE= 1,2-*bis*(2,4,6-tribromophenoxy) ethane, DBDPE= Decabromodiphenylethane.

4.3.3 Concentration of PBDEs and NBFRs on polypropylene and glass samples

A total of 16 samples from Month 3, 15 from Month 9 and 7 from Month 12 were processed and analysed for PBDEs and NBFRs in this study (Table 4.7). The frequency of detection and concentrations of PBDE and NBFRs on PP, PLA and glass coupons recovered from the Yarra River from this study are displayed in Table 4.8. The frequency of detection of the PBDE congeners over the 12 month exposure experiment from highest to lowest was penta-BDEs (BDE-99 (95%) and BDE-100 (95%)) , hexa-BDE (BDE-153 (92%) and BDE-154 (89%)), tetra-BDE (BDE-47 (79%)), hepta-BDE (BDE-183 (68%)), deca-BDE (BDE-209 (55%)) and tri-BDE (BDE- 28 (29%)). The frequency of detection of the NBFR compounds over the 12 month exposure experimentfrom highest to lowest was PBT (84%), HBB (74%), PBEB (24%) and DBDPE (11 %).

Table 4.7 Samplesused for the analysis of PBDEs and NBFRs on polylactic acid (PLA), polypropylene (PP) and glass substrates. Samples that underwent GC-MS analysis are shown with respect to each sampling date, sample site and substrate type.

		Sampling date		
		Month 3 (n=16)	**Month 9 (n=15)**	**Month 12 (n=7)**
Sites	**Dights Falls**	4	7	7
	MacRobertson Bridge	4	5	N/D
	Federation Square	1	N/D	N/D
	Westgate Bridge	3	N/D	N/D
	Williamstown	4	3	N/D
Substrate type	**PLA**	N/D	8	5
	PP	11	5	2
	Glass	5	2	N/D

PBDEs= Polybrominated diphenyl ethers (PBDEs), NBFRs= Novel brominated flame retardants, n= Total sample size per sampling date, N/A= not determined.

At least one PBDE congener and NBFR compound were detected on all samples. The sum total of the frequencies that PBDEs congeners and NBFRs compounds that were detected but not quantified increased from Month 3 (66% and 34%, respectively) to Month 12 (84% and 66%, respectively). The sum total of the frequency of quantifiable PBDE congeners increased from Month 3 (14%) to Month 12 (30%). NBFR compounds were only quantifiable on two samples (samples M3S2P2 and M12S1L3). The PLA sample M12S1L3 had the highest sum of PBDE and NBFR concentrations, with a ∑PBDE concentration of 270 ng g^{-1}; the most concentrated PBDE conger was BDE-209(218 ng g^{-1}) and a ∑NBFR concentration of 897 ng g^{-1};the most concentrated NBFR compound was DBDPE (874 ng g^{-1}). The PLA sample M12S1L4 had the second highest ∑PBDE concentration of70 ng g^{-1}; the most concentrated PBDE congener was BDE-209 (70 ng g^{-1}), whilethe PP sample M3S2P2 had the second highest ∑NBFR concentration which was substantially lower at 0.04 ng g^{-1}; the most concentrated NBFR compound was PBEB(0.04 ng g^{-1}). The mean and median ∑PBDE concentrations were 12.3 ng g^{-1} ± 7.4 ng g^{-1} and 0.06 ng g^{-1}, respectively. The mean and median ∑NBFR concentrations were 23ng g^{-1} ± 23 ng g^{-1} and <0.001 ng g^{-1}, respectively.

Table 4.8 PBDE and NBFR frequency of detection, frequency of quantification and concentrations on PP, PLA and glass samples that were deployed in the Yarra River for up to 12 months.

				Concentration (ng g^{-1} substrate)													
				PBDEs									NBFRs				
Sampling period	**Site**	**Substrate**	**Sample**	**BDE-28**	**BDE-47**	**BDE-99**	**BDE-100**	**BDE -153**	**BDE-154**	**BDE -183**	**BDE -209**	**∑PBDE**	**PBT**	**HBB**	**PBEB**	**DBDPE**	**∑NBFR**
Month 3	**Site 1**	**Glass**	**M3S1G1**	N.D	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	N.D	N/A	<MQL	<MQL	<MQL	N.D	N/A
	Site 1	**PP**	**M3S1P1**	N.D	<MQL	<MQL	0.20	<MQL	<MQL	<MQL	N.D	0.20	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PP**	**M3S1P2**	N.D	<MQL	<MQL	0.21	<MQL	<MQL	<MQL	N.D	0.21	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PP**	**M3S1P3**	<MQL	0.28	0.39	0.49	0.32	0.19	<MQL	N.D	1.67	<MQL	<MQL	N.D	N.D	N/A
	Site 2	**Glass**	**M3S2G1**	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N.D	N/A	<MQL	<MQL	<MQL	N.D	N/A
	Site 2	**PP**	**M3S2P1**	<MQL	N.D	<MQL	N.D	<MQL	<MQL	N.D	N.D	N/A	<MQL	N.D	N.D	N.D	N/A
	Site 2	**PP**	**M3S2P2**	1.9	1.5	2.7	1.9	1.8	1.2	1.7	<MQL	12.7	<MQL	<MQL	0.04	N.D	0.04
	Site 2	**PP**	**M3S2P3**	<MQL	0.97	1.2	0.42	<MQL	<MQL	N.D	N.D	2.59	N.D	N.D	N.D	N.D	N/A
	Site 3	**Glass**	**M3S3G1**	N.D	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	N.D	N.D	N/A
	Site 4	**Glass**	**M3S4G1**	N.D	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	N.D	N.D	N/A
	Site 4	**PP**	**M3S4P1**	N.D	N.D	<MQL	N.D	N.D	N.D	N.D	<MQL	N/A	N.D	N.D	N.D	N.D	N/A
	Site 4	**PP**	**M3S4P2**	N.D	<MQL	<MQL	<MQL	N.D	N.D	<MQL	<MQL	N/A	N.D	N.D	N.D	N.D	N/A
	Site 5	**Glass**	**M3S5G1**	<MQL	0.06	<MQL	N.D	<MQL	<MQL	N.D	N.D	0.06	<MQL	N.D	N.D	N.D	N/A
	Site 5	**PP**	**M3S5P1**	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N.D	N.D	N/A	<MQL	N.D	N.D	N.D	N/A
	Site 5	**PP**	**M3S5P2**	<MQL	<MQL	<MQL	<MQL	N.D	<MQL	N.D	N.D	N/A	N.D	N.D	N.D	N.D	N/A
	Site 5	**PP**	**M3S5P3**	<MQL	N.D	N.D	<MQL	N.D	N.D	N.D	N.D	N/A	N.D	N.D	N.D	N.D	N/A
Month 9	**Site 1**	**Glass**	**M9S1G1**	N.D	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	N.D	N/A	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PLA**	**M9S1L1**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	<MQL	N.D	N/A
	Site 1	**PLA**	**M9S1L2**	N.D	0.50	<MQL	0.70	<MQL	<MQL	<MQL	<MQL	1.2	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PLA**	**M9S1L3**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	<MQL	N.D	N/A
	Site 1	**PP**	**M9S1P1**	N.D	0.20	0.21	0.40	0.13	<MQL	<MQL	N.D	0.94	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PP**	**M9S1P2**	N.D	0.25	0.21	0.39	<MQL	N.D	<MQL	N.D	0.85	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PP**	**M9S1P3**	N.D	0.32	N.D	0.49	0.12	<MQL	<MQL	N.D	0.93	<MQL	<MQL	N.D	N.D	N/A
	Site 2	**Glass**	**M9S2G1**	N.D	<MQL	<MQL	0.06	<MQL	<MQL	<MQL	<MQL	0.06	<MQL	<MQL	N.D	N.D	N/A
	Site 2	**PP**	**M9S2P1**	N.D	0.35	0.30	0.49	0.25	0.19	N.D	N.D	1.58	<MQL	<MQL	N.D	N.D	N/A
	Site 2	**PP**	**M9S2P2**	N.D	N.D	N.D	0.49	<MQL	N.D	N.D	N.D	0.49	<MQL	N.D	N.D	N.D	N/A
	Site 2	**PLA**	**M9S2L1**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	<MQL	N.D	N/A
	Site 2	**PLA**	**M9S2L2**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	N.D	N.D	N/A
	Site 5	**PLA**	**M9S5L1**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	45	45	<MQL	<MQL	N.D	<MQL	N/A
	Site 5	**PLA**	**M9S5L2**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	N.D	<MQL	N/A
	Site 5	**PLA**	**M9S5L3**	N.D	<MQL	<MQL	0.26	<MQL	<MQL	N.D	<MQL	0.26	<MQL	<MQL	N.D	N.D	N/A
Month 12	**Site 1**	**PLA**	**M12S1L1**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	N/A	<MQL	<MQL	<MQL	N.D	N/A
	Site 1	**PLA**	**M12S1L2**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	N.D	<MQL	N/A	N.D	N.D	N.D	<MQL	N/A
	Site 1	**PLA**	**M12S1L3**	8.1	8.2	8.6	8.7	9.3	8.9	N.D	218	270	7.4	8.0	7.6	874	897
	Site 1	**PLA**	**M12S1L4**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	70	70	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PLA**	**M12S1L5**	N.D	<MQL	<MQL	<MQL	<MQL	<MQL	N.D	55	55	<MQL	<MQL	N.D	N.D	N/A
	Site 1	**PP**	**M12S1P1**	N.D	0.36	0.25	0.45	0.17	<MQL	<MQL	N.D	1.23	<MQL	<MQL	<MQL	N.D	N/A
	Site 1	**PP**	**M12S1P2**	<MQL	0.52	0.47	0.69	0.33	0.24	<MQL	<MQL	2.25	<MQL	<MQL	N.D	N.D	N/A
Frequency of detection of analytes over 12 month experimental period (%)				**29**	**79**	**95**	**95**	**92**	**89**	**68**	**55**	**N/A**	**84**	**74**	**24**	**11**	**N/A**
Frequency of quantification of analytes over 12 month h experimental period (%)				**5.1**	**31**	**26**	**41**	**23**	**13**	**2.6**	**10**	**N/A**	**2.6**	**2.6**	**5.1**	**2.6**	**N/A**

PBDEs = Polybrominated diphenyl ethers, NBFRs = Novel brominated flame retardants, N.D = Not detected, MQL = Method quantitation limit. Concentrations >MQL are in red text, N/A = Not applicable, BDE-= Polybrominated diphenyl ether congener, PBT = Pentabromotoluene, HBB = Hexabromobenzene, PBEB = Pentabromoethylbenzene, DBDPE = Decabromodiphenylethane.

4.4 Discussion

The aim of this study was to investigate the potential for PBDEs and NBFRs to accumulateonto PLA, PP and glass substrates exposed in surface waters along a freshwater-marine continuum of the Yarra River, Melbourne, over a 12-month period. To achieve this, a novel extraction and processing method was successfully developed that allowed for the detection and quantification of PBDEs and NBFRs from PLA substrates.

The analysed PBDEs and NBFRs were detected on all coupon samples. The most frequently detected PBDE congeners on the coupons were BDE-99 and BDE-100 which were detected in 95% of the samples. Both congeners are from the penta-BDE commercial formulationswhich have never been produced in Australia (National Industrial Chemicals Notification and Assessment Scheme (NICNAS), 2007), and their production has been banned in many countries for approximately a decade (United Nations Environment Programme, 2009, Stapleton *et al.*, 2012). Therefore, the high prevalence of BDE-99 and BDE-100 is somewhat alarming and may be due to legacy compounds in the environment or new releases from products into the environment. BDE-209 was detected in just over half of the samples in this study (53%). BDE-209 was the main BDE congener in the deca-BDE technical formulations, production of which was only recently banned under the Stockholm Convention in 2017 (United Nations Environment Programme, 2017). Therefore, while such high detection rates are of concern, they are not as alarming as those of BDE-99 and BDE-100. The most commonly detected NBFR was PBT, being detected in 84% of samples. This is in contrast with previous studies from Melbourne that have found PBT to be the least prevalent NBFR(including HBB, BTBPE and DBDPE) in soils and similarly in house-hold dust samples (McGrath *et al.*, 2017b, McGrath *et al.*, 2018). PBTs in soilsfrom nearto waste

disposal facilities had a maximum concentration of 0.1 ng g^{-1} dw (McGrath *et al.*, 2017b), and in household dust a maximum concentration of 8.0 ng g^{-1}(McGrath *et al.*, 2018). Of the four NBFRs that were detected and quantified in this study, PBT had the highest water solubility of 7.80 x 10^{-4} g L^{-1} (25 ^{o}C), which could explain why it had the highest frequency of detectionin the river environment when compared to DBDPE which had the lowest frequency of detection (11%) and also the lowest water solubility of 2.10 x10^{-7} g L^{-1} (25 ^{o}C) (Table 4.1).

The PBDE congener detected at the highest concentration was BDE-209 (218 ng g^{-1}). This congener was only quantifiable at levels above the MDLs and MQLs in samples at Month 9 (Williamstown) and Month 12 (Dights Falls) (both samples were PLA). BDE-209 is consistently found to be the most prevalent PBDE in other environmental samples such as car dust (Harrad *et al.*, 2008, Gevao *et al.*, 2016, Besis *et al.*, 2017), blood serum (Sales *et al.*, 2017, Guo *et al.*, 2018) and in soils (Cheng *et al.*, 2014, Li *et al.*, 2018). DBDPE was detected at the highest concentrations (874 ng g^{-1}) among the NBFRs and was again only detected in the Month 9 and Month 12 samples (on PLA coupons). DBDPE is a common NBFR and is produced as a replacement for BDE-209 (Vorkamp *et al.*, 2015); BDE-209 and DBDPE are structurally similar, with both rings being fully brominated (Figure 4.1).

The upper PBDE concentration in this current studywas 270 ng $g^{-1}$and the upper NBFR concentration was 897ng g^{-1}. These concentrationsweresimilar to those reported in previous studies which have investigated PBDEs on plastic debris recovered from the Central and North Pacific Gyres (Hirai *et al.*, 2011, Chen *et al.*, 2018). However, the median concentration of the ∑PBDEsfrom this study (0.06 ng g^{-1}) was two orders of magnitude lower than that of the previous studies (3.36 ng g^{-1};Hirai *et al.* (2011) and 6.1 ng g^{-1};Chen *et al.* (2018)). The plastics analysed by Hirai *et al.* (2011) and Chen *et al.* (2018)were likely present

in the seawater for longer than 12 months and therefore had much a longer time to accumulate the BFRs. Similarly, the concentration of PCBs and PAHs on plastic debris in marine environments has tended to be lower in exposure studies which have been conducted over a 12-month period when compared to plastic fragments that have been sourced directly from the environment (see Section: 1.7.1). For instance, PCBs were quantified within a range of 1 ng g^{-1}-223 ng g^{-1} from plastic recovered from the North Pacific Gyre (Rios Mendoza and Jones, 2015) and within a range of 8ng g^{-1}-34 ng g^{-1} from PE pellets that had been deployed in the San Diego Bay, USA, for a 12 month period (Rochman *et al.*, 2013a). Although the time to equilibrium for the adsorption of hydrophobic organic chemicals (HOCs) onto plastics in marine environments is estimated to be in the order of months (Koelmans *et al.*, 2016), these estimates are based on pristine plastic, and do not factor for changes in surface hydrophobicity due to biofilm formation and weathering. Plastic pellets are also known to become coloured and darker over time when exposed to water (Rochman *et al.*, 2013a), and coloured and dark plastic pellets tend to have higher concentrations of PCBs when compared to white pellets (Ogata *et al.*, 2009, Antunes *et al.*, 2013, Fisner *et al.*, 2017). This may be because the aged weathered pellets have more fractures and an increased surface area (Van *et al.*, 2012). No such colourisation occurred for the plastics in this current study.

The glass slides were used as a non-plastic substrate and it was expected that PBDEs and NBFRs would adsorb less frequently and at lower concentrations onto the glass compared to the plastic substrates due to the PBDE and NBFR having high octanol-water partitioning coefficients (Table 4.1). However, PBDEs and NBFRs were also detected on the glass slides. As all three substrate types were heavily biofouled by Month 3 (see Section 3.3.4), it is possible that the PBDEs and NBFRs detected were associated with the biofilm directly or adsorbed to sediment particles, in which they are known to be ubiquitous(de Wit, 2002), and

which were caught in the biofilm matrix. A previous study comparing the concentration of trace metals between the surface of plastic pellets and the associated biofilm fraction found that most of the metals were held in the biofilms and were not associated directly with the plastic surface(Ashton *et al.*, 2010). To date no such comparison has been made for POPs.

To conclude, in this chapter it was shown over a 12-month period that PBDEs and NBFRs from the surrounding water frequently accumulated onto PLA, PP and glass substrates at high frequencies (up to 95% detection rate). Most detected PBDEs and NBFRs that were detected on the PLA, PP and glass substrates were presentat levels below 10 ng g^{-1}, although some coupons had PBDEs and NBFRs in the order of hundreds nanogram per gram of substrate (ng g^{-1}). A novel analytical method was developed to isolate and enable quantification of PBDEs and NBFRs analytes from PLA substrates. For the first time, bioplastics such as PLA have been shown to have the capacity to accumulate POPs, and therefore ingested bioplastics may pose a similar level of risk to aquatic biotafor exposure to hydrophobic organic chemicals (HOCs) as has been suggested for petroleum-based plastics and ultimately facilitate POP bioaccumulationin natural aquatic environments.

Chapter 5: Variation in microbial community structure and composition in biofilms on plastics and bioplastics along a freshwater-marine continuum

5.1 Introduction

Microbial biofilm communities on plastic debris in aquatic environments have been shown to be structurally and functionally distinct from the free-living communities in surrounding aquatic environments (McCormick *et al.*, 2016, Arias-Andres *et al.*, 2018, Dussud *et al.*, 2018), with diverse microbial biofilm communities varying with spatial (Oberbeckmann *et al.*, 2014), temporal (Pollet *et al.*, 2018) and seasonal (Oberbeckmann *et al.*, 2016) factors. Plastic debris biofilm communities have also been found to be distinct in their structure when compared to those on organic-particulate matter (>3 μm) in marine environments (Dussud *et al.*, 2018), and from those on leaves and cardboard in freshwater environments (Hoellein *et al.*, 2014). When assessing community composition at either the phylum, class or family taxonomic levels in aquatic systems, significant differences in overall community structure between plastic-biofilm communities and biofilms on other hard-substrates, such as glass, have not been reported (Hoellein *et al.*, 2014, Oberbeckmann *et al.*, 2016). This suggests that biofilm formation on plastic substrates follows conventional biofilm formation processes (Dang and Lovell, 2016),although minor but not significant differences in plastic hard-substrate biofilm community compositions have been observed when assessing the microbial communities at the family taxonomic level (Oberbeckmann *et al.*, 2016). Oberbeckmann *et al.* (2016) observed that taxa within the Cryomorphaceae and Alcanivoraceae families increased in their relative abundance on poly(ethylene terephthalate) (PET) when compared to communities on glass substrates in the North Sea, over a 5-6 week period. There is also

limited evidence to suggest that bacterial biofilm community structure on plastics converge over time (Harrison *et al.*, 2014), although, this study was conducted using laboratory based microcosms as opposed to field exposure and additionally over a relatively short time (14 day) period, and hence may not reflect community dynamics under *in situ* conditions and over longer durations.

Marine microplastics have been found to be sites of relatively high microbial primary production, with spherical 5 mm microplastics estimated to have chlorophyll-α concentrations equivalent to those of the production occurring within 30 mL to 700 mL of seawater (Bryant *et al.*, 2016). It has been hypothesised that marine plastic debris may pose a risk of facilitating an increase in pathogen dispersal in aquatic environments, including of *Vibrio spp*., although this was from an observation from only one sample (Zettler *et al.*, 2013). Nevertheless, evidence is building in support of this hypothesis. Recently, Viršek *et al.* (2017) observed a fish pathogen, *Aeromonas salmonicida*, for the first time on plastic debris in the Mediterranean Sea. Furthermore, marine plastic debris may be a mode of dispersal for species beyond their natural domains (Gregory, 2009, Miralles *et al.*, 2018). However, the magnitude of potential species dispersal by floating plastic debris is not yet known when compared with other known methods of species transfer such as through the ornamental fish trade or ship ballast water (Rahel, 2007) or via transport on pumice stone subsequent to volcanic eruptions (Bryan *et al.*, 2012).

The body of research into plastic-degrading organisms has predominately relied on laboratory studies, based on this review of the current literature. To date,75bacterial and fungal taxa have been identified with the ability to potentially degrade various types of plastic (see also Section1.5.2). However, these studies have relied on microcosm-based experimental designs to investigate degradation. Therefore, there is a gap in our knowledge regarding the

occurrence of these potential plastic-degrading organisms on plastic debris in natural aquatic environments.

Previous studies of plastic biofilms in aquatic environments have focused on petroleum-based plastics such as polypropylene (PP) polystyrene (PS), and poly(ethylene terephthalate) (PET), ignoring bioplastics. In Chapter 3, polylactic acid (PLA) which is the most highly produced bioplastic;European Bioplastics (2013) was shown to be as resilient as PP following exposure in aquatic environments for up to one year. Therefore, with previous research into plastic biofilm formation not considering bioplastics as a substrate for biofilm formation, a substantial gap in our knowledge remains in relation to bioplastic microbiome community structure and composition.

The experimental design used in previous research into plastic-biofilms has largely been divided between studies which analyse plastics directly recovered from the aquatic environment and those involving controlled exposure experiments or microcosm studies (Table 5.1). An advantage of using plastic debris sourced directly from the environment is that samples recovered from across large geographical areas and from diverse environments can be investigated. However, the microbial colonisation processes on the plastics and the period of time for which they have been in the source environment remains unknown. An advantage of the controlled exposure experiments is that data can be generated at multiple time points during the colonisation process on plastics and the period in which the plastic is in water environments is known. However, exposure studies to date have been conducted over a relatively short time-frame of weeks (Harrison *et al.*, 2014, Hoellein *et al.*, 2014, McCormick *et al.*, 2014, Kettner *et al.*, 2017), compared to the expected multi-decade residence time of plastic in aquatic environments.

To date, most of the research into plastic biofilm communities has predominately focused on the marine environment (Table 5.1) and to a far lesser extent on freshwater environments, and in turn, estuarine waters. Estuarine environments under the influence of changing salinities can have distinct microbial communities when compared to those in either marine or freshwater environments (Bernhard *et al.*, 2005, Crump *et al.*, 2004, Sun *et al.*, 2014). Therefore, there is a substantial gap in our knowledge of the development of microbial biofilms on plastics in and along estuarine systems.

Table 5.1 Studies investigating aquatic plastic biofilm community structure and composition from marine and freshwater environments. N/A indicates data not available.

Environment	Water/ sediment samples	Experiment type	Duration of polymer exposure	Plastic substrate type	Additional substrates	Analytical technique	Key bacteria in plastic biofilms	Key eukaryotes in plastic biofilms	Key findings	Reference
Freshwater	N/A	Exposure experiment	52 days	PET	Aluminium, ceramic tiles, glass, cardboard and leaves	High-throughput sequencing	Proteobacteria, Bacteroidetes and Firmicutes	N/A	Community composition was similar between the plastic and hard substrates (aluminium, ceramic, and glass), but different when compared to the soft substrates (cardboard and leaves).	(Hoellein *et al.*, 2014)
Freshwater	Freshwater	Field sampling	N/A	N/A	Organic matter	High-throughput sequencing	Pseudomonadaceae, unclassified Proteobacteria and Campylobacteraceae	N/A	Plastic biofilm communities were less diverse compared to those in free-living water communities.	(McCormick *et al.*, 2014)
Freshwater	Freshwater	Field sampling	N/A	N/A	Organic particulate matter	High-throughput sequencing	Pseudomonadaceae, Burkholderiales, Veillonellaceae and Campylobacteraceae	N/A	Plastic biofilm communities had lower species richness, diversity and evenness compared to those on other organic matter substrates and in free-living picoplankton communities	(McCormick *et al.*, 2016)
A freshwater-estuarine-marine gradient	Water	Exposure experiment	14 days	PS and PE	Wood pellets and organic particulate matter	High-throughput sequencing	Flavobacteriaceae, Rhodobacteraceae and Methylophilaceae	N/A	Hyphomonadaceae and Erythrobacteraceae were present at elevated relative abundance on plastic biofilms when compared to wood biofilms, particle attached and free-living picoplankton.	(Oberbeckmann *et al.*, 2018)
Marine sediments	Sediments	Laboratory-based microcosm	14 days	PE	N/A	Terminal-restriction fragment length polymorphism (T-RFLP), cloning and sequencing	*Arcobacter spp.* and *Colwellia spp.*	N/A	Biofilm bacterial communities on PE converged by day 14.	(Harrison *et al.*, 2014)
Marine sediment and beached plastic	Seawater and sediment	Field sampling	N/A	N/A	N/A	High-throughput sequencing	Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes	N/A	Marine sediment plastic biofilms had higher diversity and species richness than in the free-living water communities but lower diversity than in the sediment biofilm communities.	(De Tender *et al.*, 2015)
Beaches	N/A	Field sampling	N/A	PET	Fishing gear	DNA sequencing-analysis using nBLAST	N/A	*Lepasspp.* (Gooseneck barnacles)	Invasive species; *Austrominius modestus, Magallana gigas*, and *Amphibalanus amphitrite* were identified on beached plastics.	(Miralles *et al.*, 2018)
Marine water	Seawater	Field sampling	N/A	PE and PP	N/A	High-throughput sequencing	Rhodobacteraceae, Anaerolinaceae and Hyphomonadaceae	N/A	A relatively high number of *Vibrio spp.*were identified on one sample. Note: only six plastic samples were analysed.	(Zettler *et al.*, 2013)
Marine water	Seawater	Exposure experiment	6 weeks	PET	N/A	Denaturing gradient gel electrophoresis and rRNA gene sequencing	Bacteroidetes, Proteobacteria and Cyanobacteria	Bacillariophyceae and Phaeophyceae	Spatial and seasonal variation in PET biofilm communities was observed.	(Oberbeckmann *et al.*, 2014)
Marine water	N/A	Field sampling	N/A	PS, PE, PP and PET	Aluminium, chitin and paint	Denaturing gradient gel electrophoresis and ABI 3130XL sequencer	Cyanobacteria	N/A	Spatial and seasonal variation in biofilm communities.	(Oberbeckmann *et al.*, 2014)
Marine water	N/A	Field sampling	N/A	PS, PE and PP	N/A	Scanning electron microscopy	N/A	Diatoms bryozoans and barnacles	Pits and grooves in plastic surfaces were identified as potentially being associated with microorganisms.	(Reisser *et al.*, 2014)
Marine water	Seawater	Field sampling	N/A	N/A	N/A	High-throughput sequencing	Cyanobacteria, Alphaproteobacteria, and Bacteroidetes	Bryozoa	*nifH* genes had relatively higher abundances in plastic biofilm communities when compared to the free-living picoplankton communities, suggesting plastics were sites of enriched nitrogen fixation.	(Bryant *et al.*, 2016)

Marine water	Seawater	Field sampling	N/A	PE, PET, PS	Wood	High-throughput sequencing	Alphaproteobacteria Streptomycetales and Cyanobacteria	Alveolata, Viridiplantae, Stramenopiles and Fungi	Differences in community composition between plastic fragment sizes were observed. Only one OTU was identified as *Vibrio sp.*.	(Debroas *et al.*, 2017)
Marine water	Seawater	Field sampling	N/A	N/A	N/A	Sanger sequencing	Alphaproteobacteria, Gammaproteobacteria, Flavobacteria and Planktomycetia	N/A	Fish pathogen *Aeromonas salmonicida* was observed.	(Viršek *et al.*, 2017)
Marine water	Seawater	Field sampling	N/A	PS, PP	Organic particulate matter	High-throughput sequencing	Alphaproteobacteria, Cyanobacteria, Gammaproteobacteria and Flavobacteriia	N/A	Plastic biofilm communities had greater species richness, diversity and evenness compared to particulate-associated and free-living picoplankton communities.	(Dussud *et al.*, 2018)
Marine water	Sediment, water, *Arenicola marina* faeces	Laboratory -based microcosm	1-5 days	PS	Glass beads	Single strand conformation polymorphism (SSCP) and Sanger-sequencing	Deltaproteobacterium and Cyanobacteria	Bacillariophyta	Enrichment of pathogens in faecal PS biofilms was not observed.	(Kesy *et al.*, 2016)
Marine water	Seawater	Exposure experiment	5-6 weeks	PET	Glass	High-throughput sequencing	Flavobacteriaceae, Cryomorphaceae and Saprospiraceae	Coscinodiscophytina and Bacillariophyta	Cryomorphaceae and Alcanivoraceae were present at elevated relative abundance on PET compared to glass substrates.	(Oberbeckmann *et al.*, 2016)
Marine water	N/A	Laboratory -based microcosm	7 days	Polyamide	Chitin	Single strand conformation polymorphism (SSCP) and Sanger sequencing	Flavobacteriaceae and *Arcobacter spp.*	N/A	Species richness was lower in the plastic biofilm communities when compared to the chitin biofilm communities.	(Kesy *et al.*, 2017)
Marine water	Seawater	Exposure experiment	15 days	PS and PE	Wood	High-throughput sequencing	N/A	Chytridiomycota, Cryptomycota and Ascomycota	Plastic biofilm communities had lower species richness, diversity and evenness compared to the wood biofilm and free-living picoplankton communities.	(Kettner *et al.*, 2017)
Marine water	Seawater	Exposure experiment	24 h	Fibreglass	N/A	High-throughput sequencing	Proteobacteria, Firmicutes and Bacteroidetes	N/A	Genes associated with quorum sensing and bacterial cell motility were detected.	(Rampadarath *et al.*, 2017)
Marine water	Seawater	Exposure experiment	75 days	PVC	N/A	High-throughput sequencing	Bacteroidetes, Alphaproteobacteria and Gammaproteobacteria	N/A	Flavobacteriia were found to be important members of the marine plastic biofilms, dominating the communities after Day 4 of the exposure study.	(Pollet *et al.*, 2018)

PP = polypropylene, PE = polyethylene, PET = poly(ethylene terephthalate), PS = polystyrene, PVC = Poly(vinyl chloride), N/A = not applicable.

Theaim of this current study was to compare spatial-, temporal- and substrate-specific (PP, PLA and glass) variation in the structure and composition of microbial (prokaryotic and eukaryotic) biofilm communitiesan in comparison to microbial communities in the surrounding waters. The study was conducted along the Yarra River, Australia, at five different sites along a freshwater, estuarine and marine water continuum over a three month period,a molecular approach (rRNA gene amplicon sequencing) was utilised. The specific aims of this study were firstly, to determine how microbial community diversity, structure and composition varied along the estuary (site variation) and over time (temporal variation). Secondly, this study sought to determine if substrate-specific communities developed over the exposure period. Thirdly, the research identified the dominant taxa present within the different substrate biofilms and in the water communities. Finally, this study investigated the presence and relative abundance of known plastic-degrading organisms, pathogenic bacteria and harmful algae in the substrate-biofilm and water communities. This new knowledge should aid in the better understanding of microbial communities which colonise both petroleum-based plastic and bioplastic pollution in aquatic environments.

5.2 Methods

5.2.1 Water filtration, DNA extraction and polymerase chain reaction (PCR) amplification

Water samples were collected from the surface of the water in sterile Schott bottles (500mL) and stored on ice during transport. Water samples (100 mL)were filtered through a 0.22 µm cellulose filter (Millipore Merck, Melbourne, Australia) within 12 h of sample collection. Filter papers were coiled using sterile tweezers and stored in 2 mL eppendorf tubes at -20 ^{o}C until further processing. DNA was extracted from the filter papers using a PowerWater® DNA Isolation Kit (MoBio laboratories, West Carlsbad, USA). For the substrates (PP, PLA and glass), biomass was removed from the substrates by stroking each side 15 times using sterile cotton swabs. This was undertaken while still in the field for the samples taken at the Month 3 time point (6th sampling event). The cotton swabs were then cut at the base and placed in Powerbead® tubes of the PowerSoil® DNA Isolation Kit (MoBio laboratories, West Carlsbad, USA) and the manufacturer's protocol was then followed. DNA extracts were kept at -80 ^{o}C. Laboratory DNA extraction blanks were conducted as the last sample to be extracted in each run using either an unfiltered cellulose filter or a sterile cotton swab for the water and substrate extracts, respectively.

Initial polymerase chain reaction (PCR) amplications were conducted on the DNA extracts using adapted primer sets suitable for use on Illumina® platforms. Bacterial and archaeal 16S small subunit (SSU) rRNA genes were targeted for amplification within the V4 hypervariable region using the universal primer pair 515F and 806R (Caporaso *et al.*, 2012). Eukaryote 18S SSU rRNA genes were targeted for amplification in the V9 region using the universal eukaryote primer pair 1391F and EukBr (Amaral-Zettler *et al.*, 2009). Primer sequences are detailed in Table 5.2. Amplification was carried out in a total volume of

25 µL containing: 1x GoTaq Flexi Buffer (Bioline, London, UK), 0.2 mM of dNTPs (Bioline, London, UK), 1.25 mM of $MgCl_2$ (Bioline, London, UK), 0.4 pmol μL^{-1} of forward and reverse primers and 1.25 U of GoTaq Polymerase (Bioline, London, UK), and 2 µL of DNA template. PCR cycling was conducted as follows: denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s and 72 °C for 90 s; and a final extension of 72 °C for 10 min (Caporaso *et al.*, 2012). PCR products were visualised, following agarose gel electrophoresis (1.2 % w/vol agarose with SYBR-safe DNA gel stain (Thermo-Fisher, Waltham, USA); in TAE buffer) under UV light at 302 nm in a Chemidoc systems (BioRad, Hercules, USA).

Table 5.2Oligonucleotide primers used in this study.

Primer name	Primer sequence (5'-3')
515F	AATGATACGGCGACCACCGAGATCTACACT-XXXXXXXXXXXX-TATGGTAATT-GT-GTGCCAGCMGCCGCGGTAA 5' Illumina adapter-Golay barcode- Forward primer pad- Forward primer linker- Forward primer (515f)
806R	CAAGCAGAAGACGGCATACGAGAT-AGTCAGCCAG-CC-GGACTACNVGGGTWTCTAAT Reverse complement of 3′ Illumina adapter- Reverse primer pad- Reverse primer linker- Reverse primer (806r)
1391F	AATGATACGGCGACCACCGAGATCTACAC-TATCGCCGTT-CG-GTACACACCGCCCGTC 5' Illumina adapter- Forward primer pad- Forward primer linker- Forward primer (1391f)
EukBr	CAAGCAGAAGACGGCATACGAGAT-XXXXXXXXXXXX-AGTCAGTCAG-CA-TGATCCTTCTGCAGGTTCACCTAC Reverse complement of 3′ Illumina adapter- Golay barcode- Reverse primer pad- Reverse primer linker- Reverse primer (EukBr)

5.2.2 Library preparation and sequencing

PCR product amplicons were purified using AMPure XP beads (Beckman Coulter, Brea, U.S.A) by following the Illumina® Nextera® DNA Library Prep Reference Guide (Illumina, San Diego, U.S.A). To allow for multiplexing of the amplicons, this protocol was further followed for the indexing PCR using the Illumina Nextera® XT index primers. Each set of amplicons were quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, U.S.A). Verification of the indexing PCR deviated from the Illumina® protocol in that analysis of PCR products using a Bioanalyzer (Agilent, Santa Clara, U.S.A) was not undertaken. Instead, a subset of 15 randomly selected amplicons were run on a 1.2 % agarose gel from amplicons before and after the indexing PCR. Indexed amplicons were normalised and then pooled to a final concentration of 4 nM, and loaded together with 15 % of PhiX. Pooled libraries were sequenced on an Illumina® MiSeq platform using Nextera® XT chemistry.

5.2.3 Processing of sequence reads

The Greenfield Hybrid Analysis Pipeline (GHAP) (Greenfield, 2015) (version 1.0) was used to classify reads and generate classified operational taxonomic units (OTUs) tables. Initially in the GHAP amplicon pipeline, split reads were demultiplexed then merged by paired reads. After paired reads were combined, a read-length histogram was created to determine where the reads should be trimmed; reads were trimmed at 250 bp. The GHAP amplicon pipeline utilises the free version of USearch which is a sequence analysis tool which produces search and clustering algorithms as well as quality controls procedures such as identifying and removing chimeric sequences (Edgar, 2017). 16S rRNA gene sequences were compared to the reference sequences on the Ribosomal Database Project (RDP)(Wang *et al.*, 2007, Cole *et al.*, 2014), 18S rRNA gene sequences were compared to the reference sequences on the SILVA database(Pruesse *et al.*, 2007).

5.2.4 Data analysis

After processing and annotating the rRNA gene sequence reads through the GHAP pipeline, the sequence data were analysed using three analysis pipelines using software packages PRIMER v7 (Clarke and Gorley, 2015), MEGAN v6 (Huson *et al.*, 2016)and the R packages vegan (Oksanen *et al.*, 2018) and iNEXT (Hsieh *et al.*, 2016) in R-Studio. After an initial square-root transformation of the OTU tables, PRIMER v7 was used to perform Analysis of Similarities (ANOSIM)(Clarke, 1993), Similarity Percentages (SIMPER)(Clarke, 1993), Permutational Multivariate Analysis of Variance (PERMANOVA)(Anderson, 2005) and non-Metric Multidimensional Scaling (nMDS) (Kruskal, 1964)plots. MEGAN v6 was used to construct Neighbor-Joining trees (Saitou and Nei, 1987)of the Bray-Curtis dissimilarity index (Bray and Curtis, 1957)and bubble plots showing the relative abundance of known taxa within the communities (species with ≥97 % I.D). The R package, vegan, was used to calculate the Shannon(Shannon and Weaver, 1949), Simpson diversity(Simpson, 1949), Pielou evenness(Pielou, 1966), nMDS plots of theBray-Curtis (Bray and Curtis, 1957) and Jaccard (Jaccard, 1901) dissimilarity indexesand rarefaction curves using the observed OTU datasets, and the R package, iNEXT, was used to calculate Chao1(Chao, 1984) species richness. Three functional guilds were screened for against three lists. The three lists were; potential plastic degrading microorganism (see Section 1.5.2); potential pathogenic bacteria (see *Appendix V*); and harmful algae (see*Appendix VI*).Pearson Correlations were tested between water physico-chemical measurements (electrical conductivity and pH (Table 2.1) and aggregated OTU Shannon diversity and OTU Chao1 richness (substrates combined for each sampling date and sampling location) (Table 5.6) using SPSS software (IBM, New York, U.S.A.). In this thesis the rare biosphere is defined as the collective microorganisms that account for < 0.01 % of 16S and 18S rRNA gene sequence reads (Lynch and Neufeld,

2015).The 20 most abundant families were calculated based on the proportion of 16S and 18S rRNA gene sequence reads assigned to them.

5.3 Results

5.3.1 Polymerase chain reaction (PCR) amplification of rRNA genes from biofilm and water DNA extracts

16S rRNA and 18S rRNA genes were amplified by PCR from biofilm coupons and from water samples at five sites along the Yarra River, Melbourne. Visualisation of example PCR products, following agarose gel electrophoresis is shown in Figure 5.1.

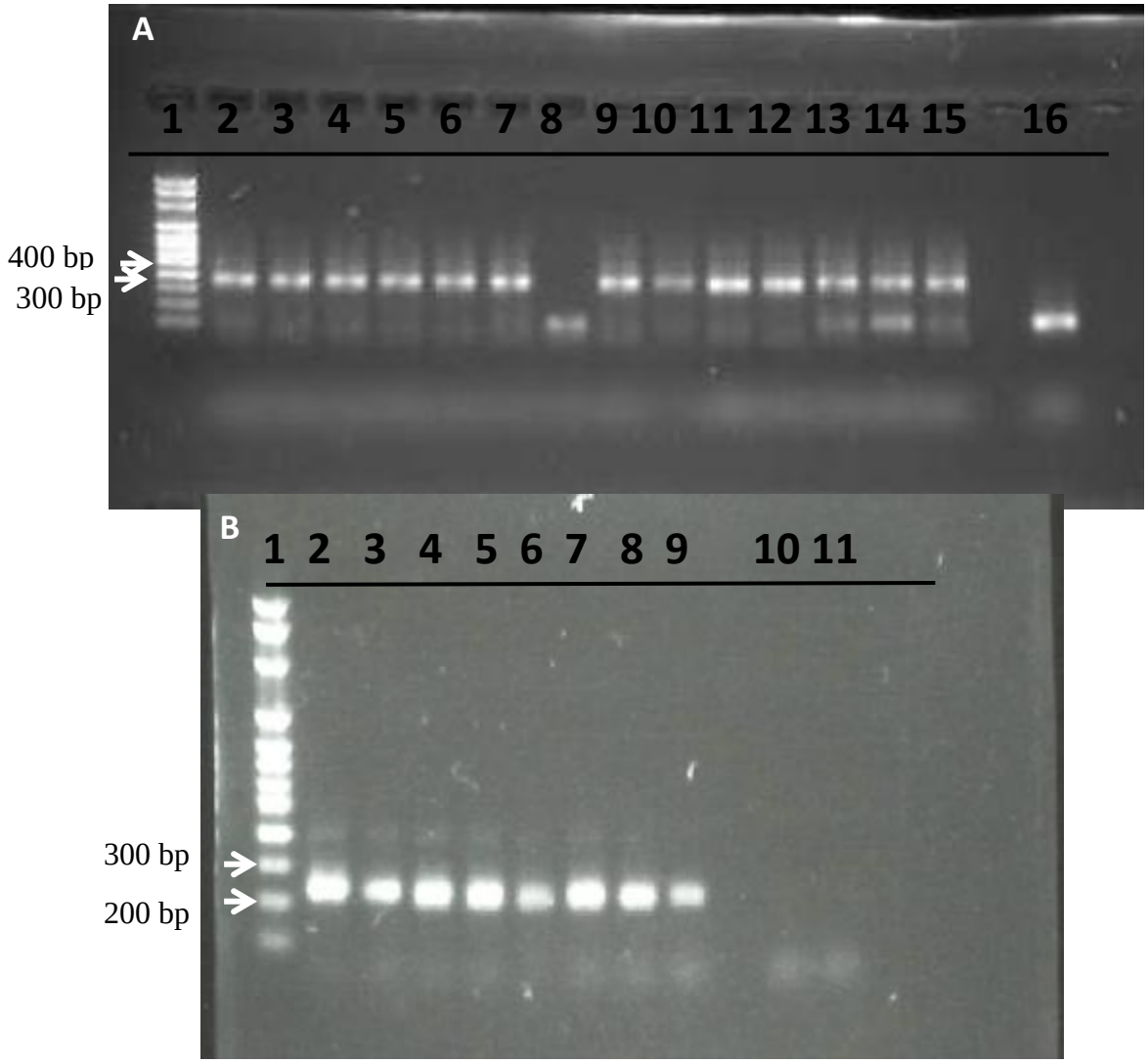


Figure 5.1Agarose gel electrophoresis (1.2% agarose) of polymerase chain reaction (PCR) amplified 16S and 18S rRNA gene sequences. A) 16S rRNA gene sequence products; 1: 100 bp ladder, 2: S1P1M3, 3: S1P2M3, 4: S1P3M3, S2G1M3, 5:S2L1M3, 6: S2L1M3, 7: S2L2M3, 8: S5G1M3, 9: S5G2M3, 10: S5G3M3, 11: S5P1M3, 12: S5P2M3, 13: S5P3M3, 14: S4L1M3, 15: S4G1M3, 16: Negative control of a DNA extraction blank. B) 18S rRNA gene sequence products; 1: 100 bp ladder, 2: S1W1D7, 3: S1W2D7, 4:S1W3D7, 5: S3W1D7,6: S3W3D7, 7: S5W1D7, 8: S5W2D7, 9: S5W3D7, 10: Negative control of a DNA extraction blank, 11: Negative control of a PCR reagents. Size of DNA markers (bp) as indicated by arrows.

5.3.2 Quality of Illumina® MiSeq DNA sequence data

DNA sequences for the 16S rRNA and 18S rRNA genes were generated for each sample from five sites along the Yarra River to Port Phillip Bay from PP, PLA and glass coupons and from water samples. Quality control statistics for DNA sequencing are given in Table 5.3. The 16S rRNA gene sequencing run was loaded with 15% PhiX control DNA, of which 7.14 % was aligned and yielded 36,110,700 reads that passed filter. The average Q30 score for the run was 70.27%. The base call error rate was below 2% for the first 200 bases for reads from each end of the amplicon (2.08% in total). For the 18S rRNA gene sequencing run a total of 33,463,968 reads passed filter. The run was loaded with 15% PhiX control, of which 6.58% was aligned. The average Q30 score for the run was 67.04%. The total error rate was 3.16% for reads from each end of the amplicon. Both sequence runs had large mean number of reads that passed filter per sample, 168,345 ± 5880 for the 16S rRNA gene sequence run and 166,525 ± 6107 for the 18S rRNA gene sequence run. Samples with fewer than 10,000 reads that passed filter were removed from subsequent analysis. These samples were: S4L3D14, S1P2D28 and BlankD14P for the 16S rRNA gene samples and S3W2D7 for the 18S rRNA gene samples.

Table 5.3 Quality control data of Illumina® MiSeq amplicon sequencing of 16S rRNA gene and 18S rRNA gene sequence runs. Variances are the standard error of the mean.

Target gene	**Total DNA sequence reads**	**Reads passed filter[a]**	**Mean* number of reads passed filter per sample**	**% ≥Q30[b]**	**Yield (Gbp)**	**Sequences Aligned to PhiX (%)[c]**	**Error rate (%)[d]**
16S rRNA gene	38,631,086	36,110,700	168,345 ± 5880	70.27	11.09	7.14	2.08
18S rRNA gene	35,374,490	33,463,968	166,525 ± 6107	67.04	10.27	6.58	3.16

*16S rRNA gene data set (n=177), 18S rRNA gene data set (n=127)

[a] An internal software quality filter of the MiSeq to remove unreliable clusters

[b] A misread of 1 in 1,000 bases.

[c] Positive control DNA

[d] Rate of miscalling an oligonucleotide base of the PhiX control DNA

The raw DNA sequences were processed using the GHAP pipeline (Greenfield, 2015). The GHAP pipeline assigns operational taxonomic units (OTUs) by clustering sequences with ≥97% sequence identity to each other. Then the OTUs are taxonomically assigned to bacterial and archaeal taxa (16S rRNA gene sequence data) with ≥97% sequence identity to taxa recorded in the RDP classifier database, or to eukaryotic taxa (18S rRNA gene sequence data) with ≥97% sequence identity to taxa recorded in the SILVA database. Rarefaction curves were generated for OTUs (Figure 5.2) and for species (≥97% identity) (Figure 5.3). The overall proportion of bacteria and archaeal OTUs that could be subsequently classified to a known species (≥97% identity) was 23.3% ± 0.5% per sample. The proportion of eukaryotes that could be classified to a known species (≥97% identity) was 9.9% ± 0.3% per sample.

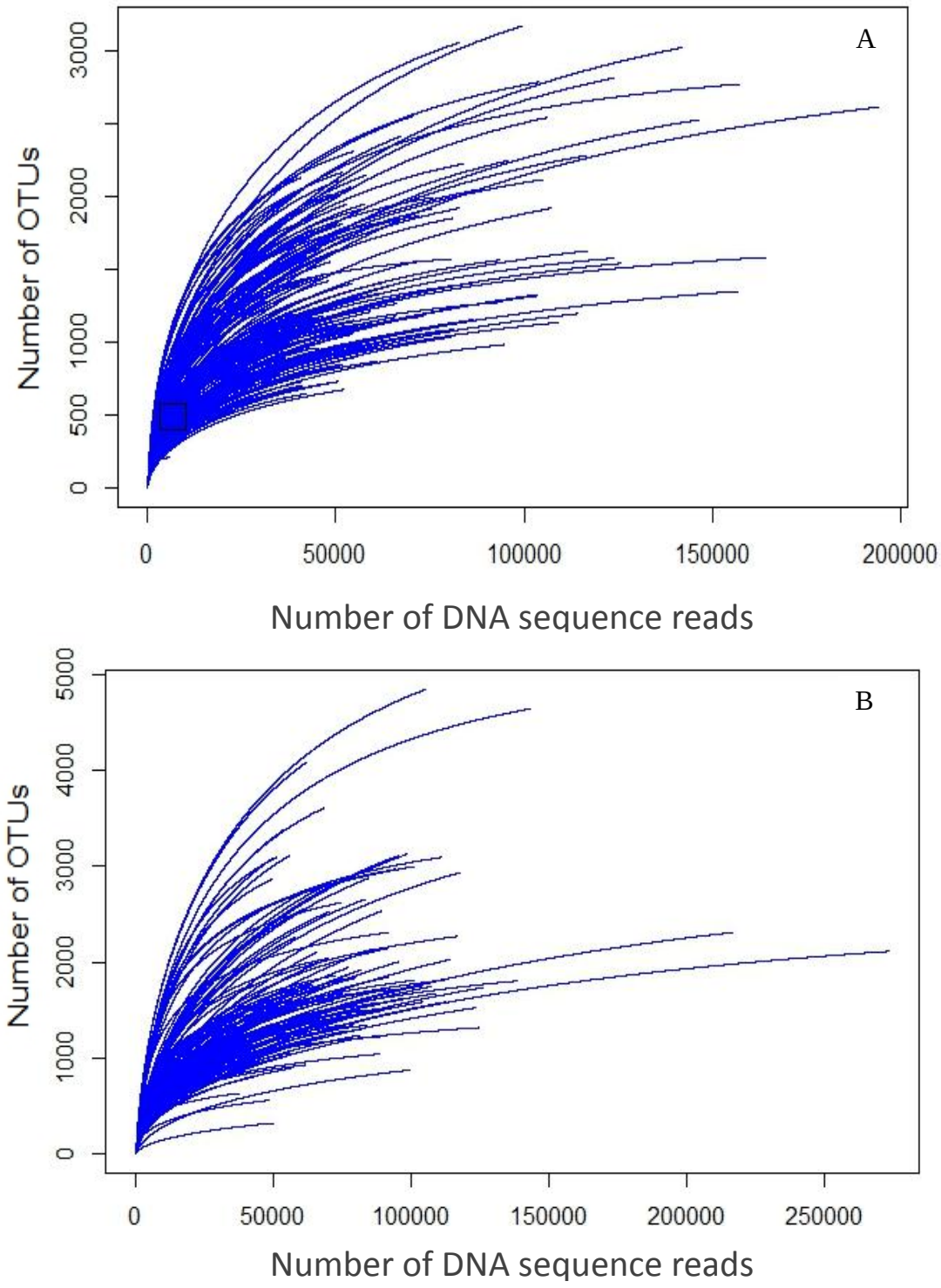


Figure 5.2 Rarefaction curves for OTUs defined at DNA sequences with ≥97% identity to each other. A) Bacterial and archaeal sequences B) eukaryotic sequences.

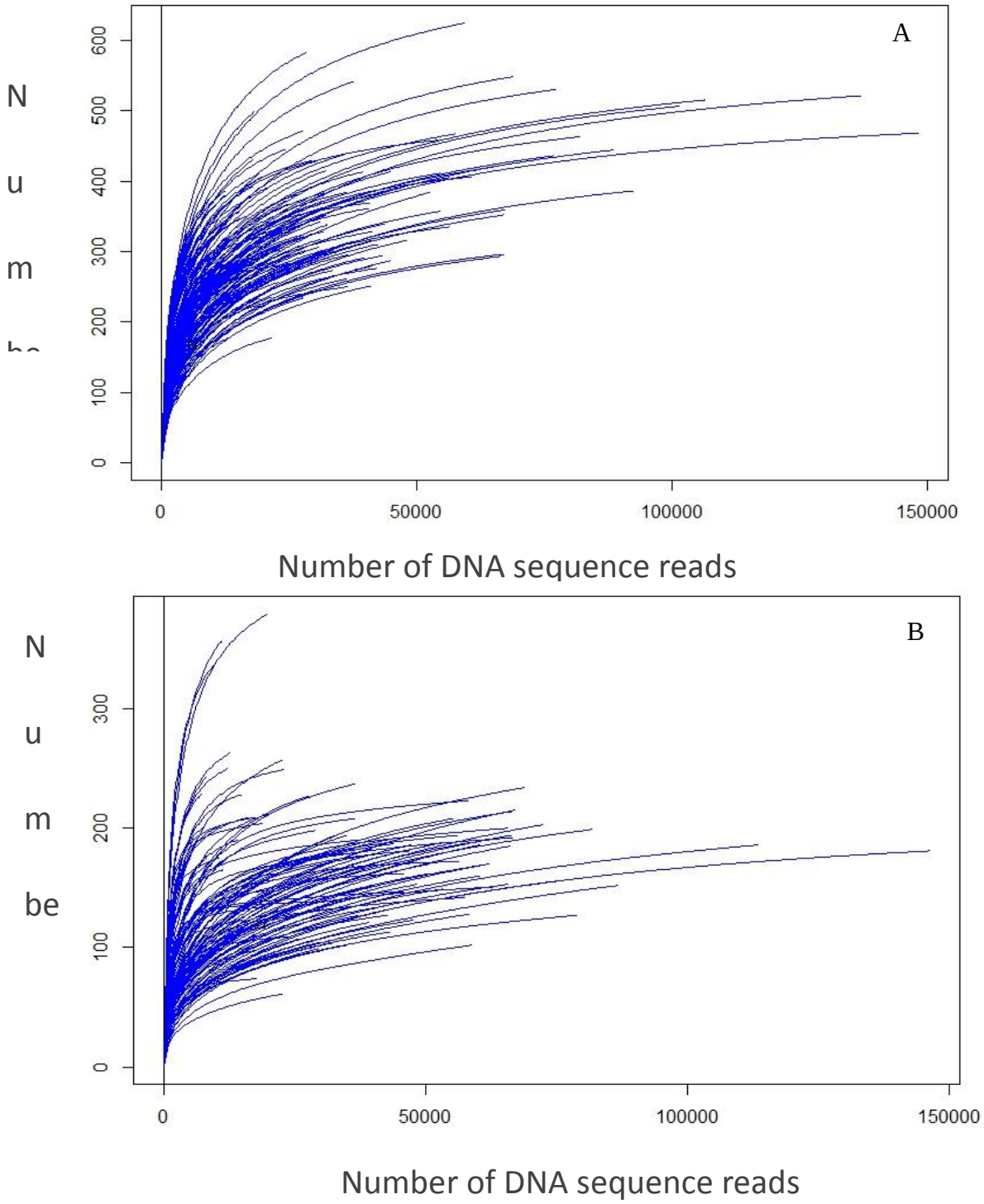


Figure 5.3 Rarefaction curves for known species with ≥97% identity to sequences from either the RDP taxonomic database for bacterial and archaeal sequences or the SILVA taxonomic database for eukaryotic sequences. A) Bacterial and archaeal sequences B) eukaryotic sequences.

5.3.3 Structure and diversity of coupon biofilm and aquatic (water) bacterial/archaeal and eukaryotic communities

5.3.3.1 Taxon richness, alpha diversity and evenness of coupon and water communities

Samples that are comprised of the coupon-biofilm communities are hereafter referred to as "biofilm communities". Water community samples which included both suspended particulate matter bound organisms and unattached organisms are hereafter referred to as "water communities". Bacteria and archaea will be collectively referred to as "prokaryotes". Species will explicitly refer to OTUs that have at least 97% identity to a known species on either the RDP or SILVA taxonomic databases.

Taxon richness, diversity and evenness were investigated for the biofilm and water communities. Observed species and OTUs, estimated Chao1 richness for species and OTUs, and OTU Shannon diversity, Simpson diversity and Pielou evenness are presented in Table 5.4 and Table 5.5 across all five sites. Overall, in both the prokaryotic and eukaryotic communities, there were more OTUs observed in the water (1,590 ± 171 and 2,400 ± 296, respectively)than in biofilms (1,420 ± 45 and 1,649 ± 62, respectively)across all five sites. Similarly, overall, the number of observed species were also greater in the prokaryotic and eukaryotic communities in water (375 ± 35 and 230 ± 16, respectively) than in biofilms (326 ± 6 and 169 ± 4, respectively).

Overall, the prokaryotic and eukaryotic communities in water had higher Shannon diversity (4.68 ± 0.09 and 5.2 ± 0.12, respectively) and Simpson diversity (0.96 ± 0.01 and 0.97 ± 0.01, respectively) than in the biofilm communities (Shannon: 4.01 ± 0.06 and 3.71 ± 0.07 Simpson: 0.91 ± 0.01 and 0.88 ± 0.01 for prokaryotes and eukaryotes, respectively). The water communities showed greater evenness than the biofilm communities (Pielou evenness: water: 0.64 ± 0.01 and 0.69 ± 0.01 biofilms: 0.56 ± 0.01 and 0.50 ± 0.01 for prokaryotes and eukaryotes, respectively).Nevertheless, there were site specific differences for the observed

OTUsand species and OTU Shannon diversity in the prokaryotic communitiesthat were not consistent with the trends highlight above. Observed OTUs, observed species and Shannon diversity were greater in the biofilm communities (1,618 ± 92, 324 ± 11, 4.77 ± 0.13, respectively) at Williamstown than in the water communities at that site (1,094 ± 58, 285 ± 24, 4.34 ± 0.25, respectively). In addition, there were no significant differences (Two-Way t-Test, $P > 0.05$) between the observed numbers of OTUsbetween the biofilm and water communities at either the Dights Falls and Westgate Bridge sites. Additionally, Shannon diversity there was not significantly different ($P > 0.05$) between the biofilm and water communities at Dights Falls.

Additionally, variation in the number of OTUs identified and the Shannon diversities in prokaryotic (Table 5.4) and eukaryotic biofilm and water communities (Table 5.5) was observed. Breifly; the number of OTUs identified and the Shannon diversities were higher in theDights Falls and Williamstown prokaryotic biofilm communities (1,868 ± 103 and 4.09 ± 0.12 and 1,618 ± 92 and 4.77 ± 0.13, respectively) than in the MacRobertson Bridge, Federation Square and Westgate Bridge communities (1,386 ± 84 and 3.72 ± 0.11, 1,104 ± 56 and 3.88 ± 0.12 and 1,113 ± 84 and 3.68 ± 0.13, respectively). In contrast, in the prokaryotic water communities, higher numbers of observed OTUs and higher Shannon diversity were observed in the MacRobertson Bridge and Federation Square communities (2,190 ± 337 and 4.9 ± 0.08 and 1,813 ± 145 and 5.18 ± 0.03, respectively) when compared to the Dights Falls, Westgate Bridge and Williamstown communities (1,621 ± 230 and 4.35 ± 0.09, 938 ± 78 and 4.59 ± 0.04 and 1,094 ± 58 and 4.34 ± 0.25 respectively). In both the biofilm and water eukaryotic communities (Table 5.5), the number of OTUs and Shannon diversity were higher in the Dights Falls (2,076 ± 115 and 3.83 ± 0.13 and 3,228 ± 290 and 5.44 ± 0.12, respectively) communities when compared to the Federation Square and Williamstown

communities (1,432 ± 64 and 3.78 ± 0.09, 1,447 ± 83 and 3.51 ± 0.15, 2,233 ± 770 and 5.17 ± 0.33 and 1,712 ± 111 and 5.0 ± 0.12, respectively).

Table 5.4 Overall estimates by community type and sample site of prokaryote observed OTUs, Chao1 Richness for OTUs, observed species, Chao1 Richness for species and OTU Shannon diversity (H'), Simpson diversity (D') and Pielou evenness (J'). Variances are the standard error of the mean ($n \geq 30$ for biofilms communities and $n \geq 5$ for water communities).

Habitat Type	Site	Observed OTUs	Chao1 OTU Richness	Observed Species	Chao1 Species Richness	OTU H'	OTU D'	OTU J'
Biofilm	**Dights Fall**	1,868 ± 103	2,164 ± 113	312 ± 13	353 ± 14	4.09 ± 0.12	0.89 ± 0.01	0.54 ± 0.01
	MacRobertson Bridge	1,386 ± 84	1,634 ± 91	363 ± 15	406 ± 15	3.72 ± 0.11	0.89 ± 0.01	0.51 ± 0.01
	Federation Square	1,104 ± 56	1,314 ± 65	344 ± 10	386 ± 11	3.88 ± 0.12	0.92 ± 0.01	0.55 ± 0.01
	Westgate Bridge	1,113 ± 84	1,321 ± 72	286 ± 14	325 ± 15	3.68 ± 0.13	0.91 ± 0.01	0.52 ± 0.01
	Williamstown	1,618 ± 92	1,833 ± 100	324 ± 11	364 ± 11	4.77 ± 0.13	0.95 ± 0.01	0.64 ± 0.01
	Mean of all sites	**1,420 ± 45**	**1,658 ± 49**	**326 ± 6**	**367 ± 6**	**4.01 ± 0.06**	**0.91 ± 0.01**	**0.56 ± 0.01**
Water	**Dights Fall**	1,621 ± 230	1,978 ± 241	297 ± 32	336 ± 35	4.35 ± 0.09	0.96 ± 0.01	0.59 ± 0.01
	MacRobertson Bridge	2,190 ± 337	2,616 ± 371	490 ± 48	552 ± 45	4.9 ± 0.08	0.97 ± 0.01	0.64 ± 0.01
	Federation Square	1,813 ± 145	2,202 ± 153	539 ± 29	608 ± 19	5.18 ± 0.03	0.97 ± 0.01	0.69 ± 0.01
	Westgate Bridge	938 ± 78	1,136 ± 97	250 ± 35	292 ± 39	4.59 ± 0.04	0.97 ± 0.01	0.67 ± 0.01
	Williamstown	1,094 ± 58	1,292 ± 49	285 ± 24	329 ± 18	4.34 ± 0.25	0.94 ± 0.02	0.62 ± 0.04
	Mean of all sites	**1,590 ± 171**	**1,914 ± 197**	**375 ± 35**	**426 ± 38**	**4.68 ± 0.09**	**0.96 ± 0.01**	**0.64 ± 0.01**
Negative control*		**420 ± 115**	**469 ± 122**	**167 ± 33**	**180 ± 29**	**3.99 ± 0.44**	**0.9 ± 0.042**	**0.68 ± 0.04**

* Negative controls were produced from DNA extraction blanks

Table 5.5 Overall estimations by community type and sample site of eukaryote observed OTUs, Chao1 Richness for OTUs, observed species, Chao1 Richness for species and OTU Shannon diversity (H'), Simpson diversity (D') and Pielou evenness (J'). Variances are the standard error of the mean ($n \geq 24$ for biofilms communities and $n \geq 5$ for water communities).

Habitat Type	Site	Observed OTUs	Chao1 OTU Richness	Observed Species	Chao1 Species Richness	OTU H'	OTU D'	OTU J'
Biofilm	**Dights Falls**	2,076 ± 115	2,699 ± 149	182 ± 4	216 ± 6	3.83 ± 0.13	0.89 ± 0.01	0.5 ± 0.01
	Federation Square	1,432 ± 64	1,858 ± 84	145 ± 6	171 ± 6	3.78 ± 0.09	0.9 ± 0.01	0.52 ± 0.01
	Williamstown	1,447 ± 83	1,775 ± 101	143 ± 7	171 ± 8	3.51 ± 0.15	0.86 ± 0.01	0.48 ± 0.02
	Mean of all sites	**1,649 ± 62**	**2,106 ± 81**	**169 ± 4**	**198 ± 4**	**3.71 ± 0.07**	**0.88 ± 0.01**	**0.50 ± 0.01**
Water	**Dights Falls**	3,228 ± 290	3,688 ± 317	211 ± 8	231 ± 6	5.44 ± 0.12	0.97 ± 0.01	0.67 ± 0.01
	Federation Square	2,233 ± 770	2,558 ± 871	254 ± 45	280 ± 46	5.17 ± 0.33	0.96 ± 0.01	0.75 ± 0.04
	Williamstown	1,712 ± 111	2,001 ± 131	227 ± 11	250 ± 14	5.0 ± 0.12	0.97 ± 0.01	0.67 ± 0.01
	Mean of all sites	**2,400 ± 296**	**2,760 ± 333**	**230 ± 16**	**254 ± 17**	**5.2 ± 0.12**	**0.97 ± 0.01**	**0.69 ± 0.01**
Negative control*		**778 ± 96**	**1,082 ± 183**	**124 ± 21**	**151 ± 31**	**4.41 ± 0.05**	**0.96 ± 0.01**	**0.66 ± 0.01**

* Negative controls were produced from DNA extraction blanks

Pearson Correlations were tested between water physico-chemical measurements (electrical conductivity and pH (Table 2.1)and aggregated OTU Shannon diversity and OTU Chao1 richness (substrates combined for each sampling date and sampling location) (Table 5.6). OTU Chao1 richness and pH were positively correlated for the prokaryotic biofilm communities ($P < 0.05$). There were no other significant correlations amongst the tested variables.

Table 5.6 Pearson Correlations between water physico-chemical measurements (electrical conductivity and pH) when compared to microbial community OTU Shannon diversity and OTU Chao1 richness. Significant results are displayed in bold.

			EC ($\mu S\ cm^{-2}$)	pH
Prokaryotic biofilm communities	**OTU H'**	Pearson	0.254	0.225
		Significance	0.279	0.44
		n	20	14
	Chao1 OTU Richness	Pearson	0.254	**0.851****
		Significance	0.297	**0.0001**
		n	20	14
Eukaryotic biofilm communities	**OTU H'**	Pearson	-0.283	0.518
		Significance	0.461	0.371
		n	9	5
	Chao1 OTU Richness	Pearson	-0.594	-0.837
		Significance	0.092	0.077
		n	9	5
Prokaryotic water communities	**OTU H'**	Pearson	-0.129	-0.466
		Significance	0.74	0.245
		n	9	8
	Chao1 OTU Richness	Pearson	-689	-0.653
		Significance	0.04	0.079
		n	9	8
Eukaryotic water communities	**OTU H'**	Pearson	-0.56	0.552
		Significance	0.248	0.334
		n	6	5
	Chao1 OTU Richness	Pearson	-0.749	-0.869
		Significance	0.087	0.056
		n	6	5

**Correlation is significant at the 0.01 level (2-tailed). n = Sample size, Pearson= Pearson Correlation, EC = Electrical conductivity, OTU H'= OTU Shannon diversity.

In the biofilm communities, the number of OTUs identified was found to vary between sampling dates (see*Appendix II*). Numbers of prokaryotic observed OTUs were higher in the Month 3 biofilm communities than in the Day 7 biofilm communities at four of the five

sampling sites; Dights Falls (M3: 2,151 ± 134; D7: 1,642 ± 195), Federation Square (M3: 1,230 ± 55; D7: 999 ± 101), Westgate Bridge (M3: 1,343 ± 111; D7: 988 ± 84) and Williamstown (M3: 2,184 ± 130; D7: 1,461 ± 115). Conversely, the number of eukaryotic OTUs increased between sampling dates (Day 7 and Month 3) at only one sampling site; Dights Falls (D7: 1,700 ± 95;M3: 2,597 ± 191). In the DNA extraction blank samples (negative controls) 420 ± 115 prokaryotic and 778 ± 96 eukaryotic OTUs were observed. Description of data from negative controls is presented in Section 5.3.9.

5.3.3.2 Microbial community structural similarity

Using the prokaryotic and eukaryotic species (≥97 % I.D) data, neighbor-joining trees based on a Bray-Curtis dissimilarity matrix were constructed. The structure of the microbial communities clustered firstly by sample location and then secondly with respect to habitat type (i.e. water vs. biofilm) (Figure 5.4 and Figure 5.5). Of the water samples taken from all five sites for the prokaryote communities, distinct communities were identified between two groups of sites; group one; Dights Falls, MacRobertson Bridge and Federation Square and group two; Westgate Bridge and Williamstown; compared to the biofilm communities which were for the most part distinct from each other at all five sites. Of the water samples taken from three sites for the eukaryotic communities, distinct communities were also identified between sites Dights Falls and Federation Square in one group and Williamstown in the other (two of the Federation Square communities grouped with the Williamstown communities); compared to the biofilm communities which were distinct at all three sites. When communities from each single site were analysed, microbial communities clustered primarilyby sampling date rather than sample substrate for both prokaryotic (Figure 5.6) and eukaryotic (Figure 5.7) communities.

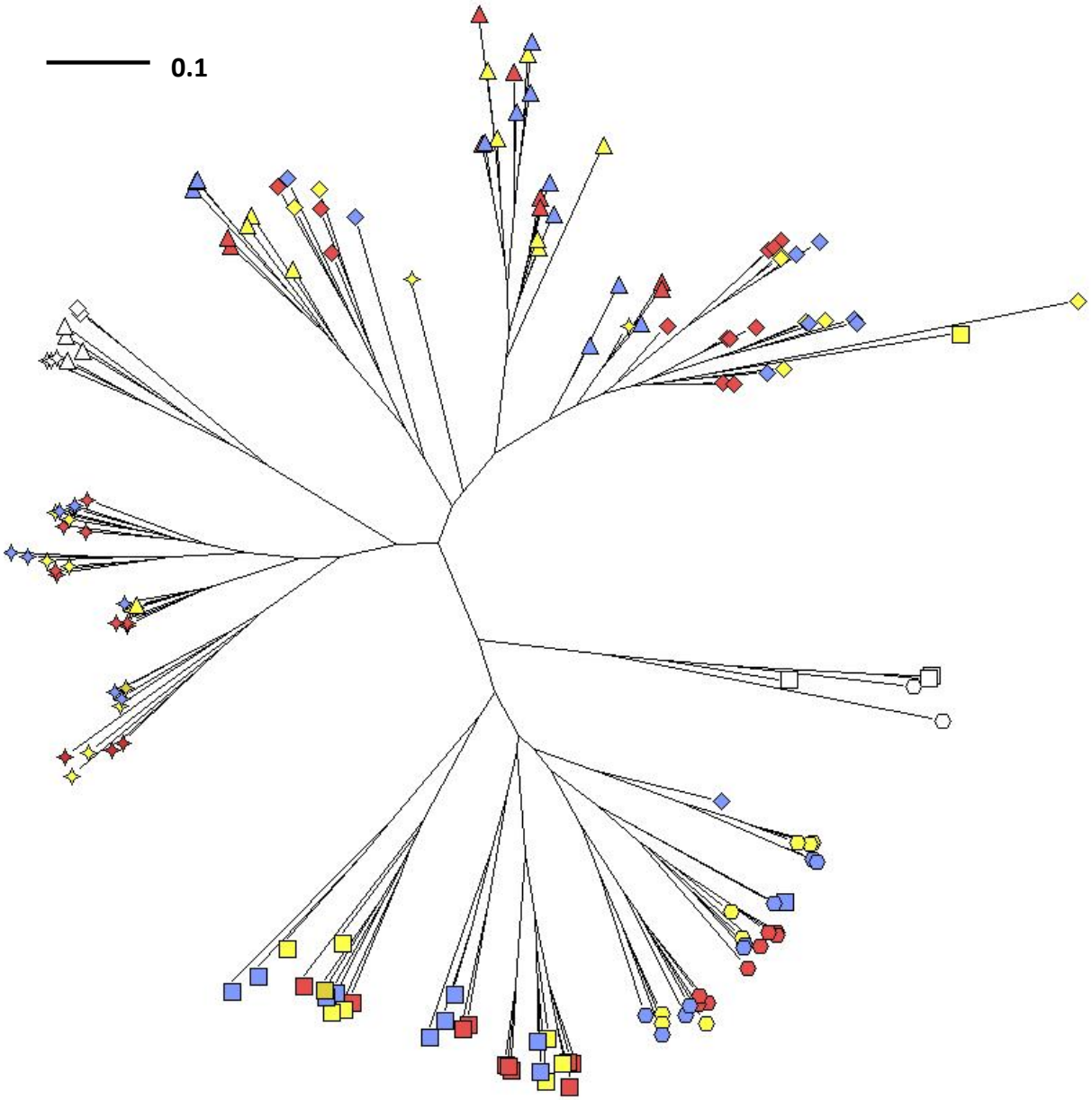


Figure 5.4 Neighbor-Joining trees showing variation in prokaryotic community structure based on a Bray-Curtis dissimilarity matrix using 16S rRNA gene sequence data. The sites are represented as shapes; Dights Falls = Stars; MacRobertson Bridge = Triangles; Federation Square = Diamonds; Westgate Bridge = Squares; Williamstown = Hexagons. Colours represent coupon type; Glass = Red; Polylactic acid (PLA) = Yellow; Polypropylene (PP) = Blue; water = White. Scale bar indicates a distance of 0.1.

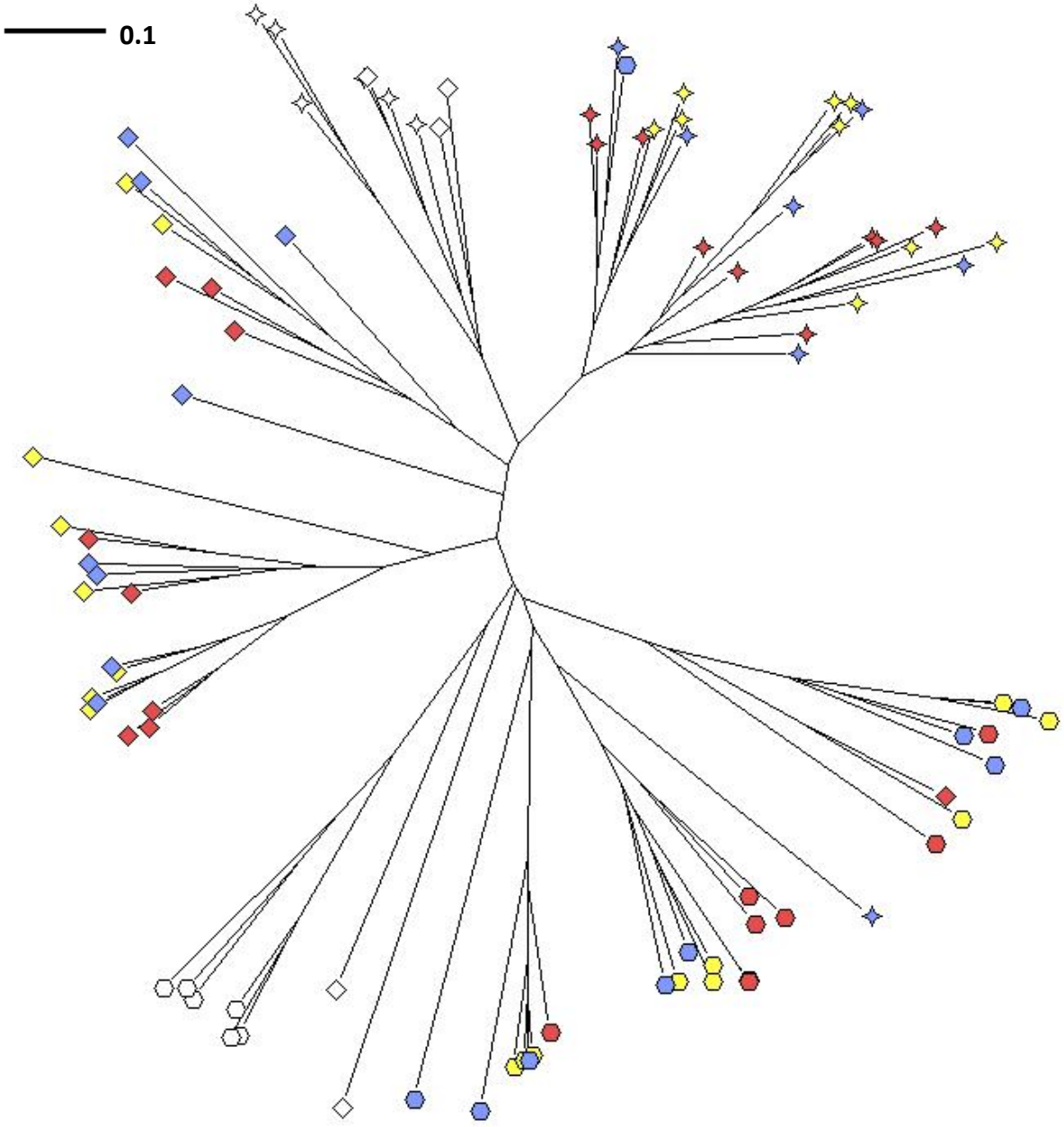


Figure 5.5 Neighbor-Joining trees showing variation in eukaryotic community structure based on a Bray-Curtis dissimilarity matrix using 18S rRNA gene sequence data. The sites are represented as shapes; Dights Falls = Stars; Federation Square = Diamonds; Williamstown = Hexagons. Colours represent coupon type; Glass = Red; Polylactic acid (PLA) = Yellow; Polypropylene (PP) = Blue; water = White. Scale bar indicates a distance of 0.1.

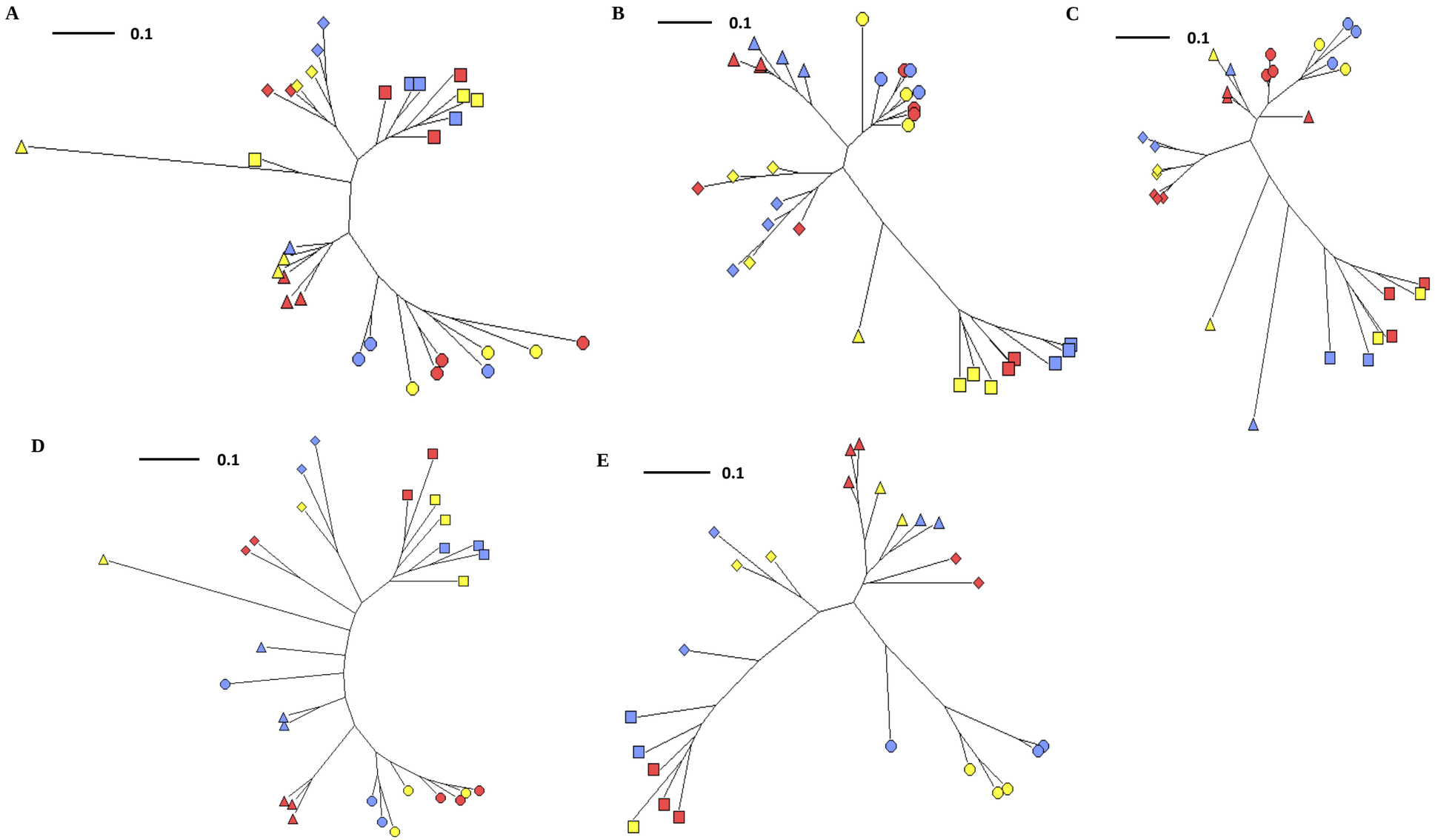


Figure 5.6 Neighbor-Joining trees showing variation in prokaryotic biofilm community based on a Bray-Curtis similarity matrix using 16S rRNA gene sequence data from A) Dights Falls; B) MacRobertson Bridge; C) Federation Square; D) Westgate Bridge; E) Williamstown. Shapes represent sampling date; Day 7 = Circles; Day 14 = Triangles; Day 28 = Diamonds; Month 3 = Squares. Colours represent coupon type; Glass = Red; Polylactic acid (PLA) = Yellow; Polypropylene (PP) = Blue. Scale bar indicates a distance of 0.1.

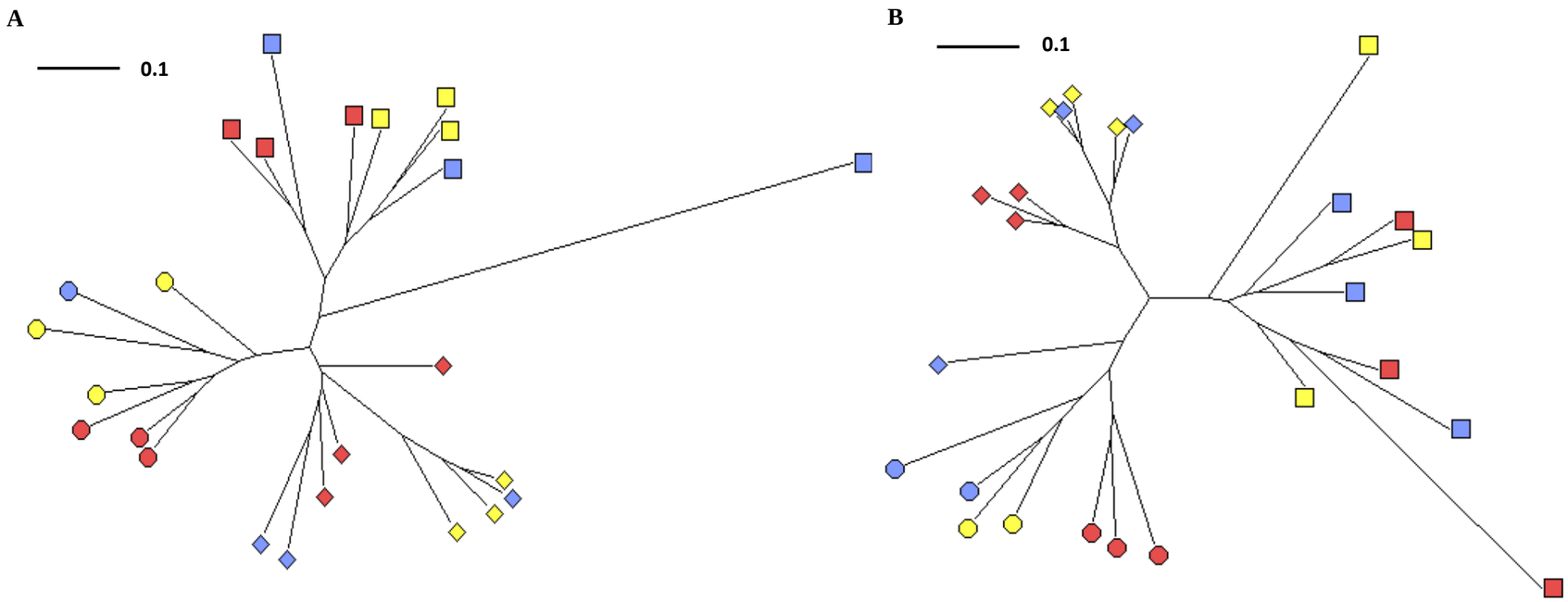


Figure 5.7 Neighbor-Joining trees showing variation in eukaryotic biofilm communities based on a Bray-Curtis similarity matrix using 18S rRNA gene sequence data from A) Dights Falls; B) Federation Square; C) Williamstown. Shapes represent sampling date; Day 7 = Circles; Day 28 = Diamonds; Month 3 = Squares. Colours represent coupon type; Glass = Red; Polylactic acid (PLA) = Yellow; Polypropylene (PP) = Blue. Scale bar indicates a distance of 0.1.

Seven biofilm samples were identified as potential outliers when compared to other samples from the same site and sampling date; these were samples S1L3D14, S2L1D14 S3L1D14 and S3P3D14 for the prokaryotic communities and S1P3M3, S3G3M3 and S5P3D7 for the eukaryotic communities. For each of these samples, DNA sequence datasets had numbers of reads which passed filter and total number of OTUs (Table 5.7) that were consistent with those samples within the larger dataset. Therefore these samples were retained in the analysis.

Table 5.7DNA sequence reads passed filter and number of OTUs from potential outlier prokaryotic and eukaryotic biofilm communities.

Community type	**Potential Outlier**	**DNA sequence reads Passed Filter[a]**	**Number of OTUs**
Prokaryotic	S1L3D14	110,499	903
	S2L1D14	138,340	1596
	S3L1D14	101,435	707
	S3P3D14	97,391	1308
Eukaryotic	S1P3M3	95,974	1151
	S3G3M3	159,857	1638
	S5P3D7	110,129	2423

[a] An internal software quality filter of the MiSeq to remove unreliable clusters.

The appropriateness of using either the Bray-Curtis dissimilarity or Jaccard dissimilarity matrixes was compared via non-metric multidimensional scaling (nMDS) plots (Figure 5.8). The Bray-Curtis dissimilarity matrix uses relative abundance data, whilst the Jaccard dissimilarity matrix uses binary presence/absence data. The plots were derived from the observed OTUs for both the prokaryotic and eukaryotic biofilm communities and were highly similar using both matrix types, showing primary separation of communities based on sample location.

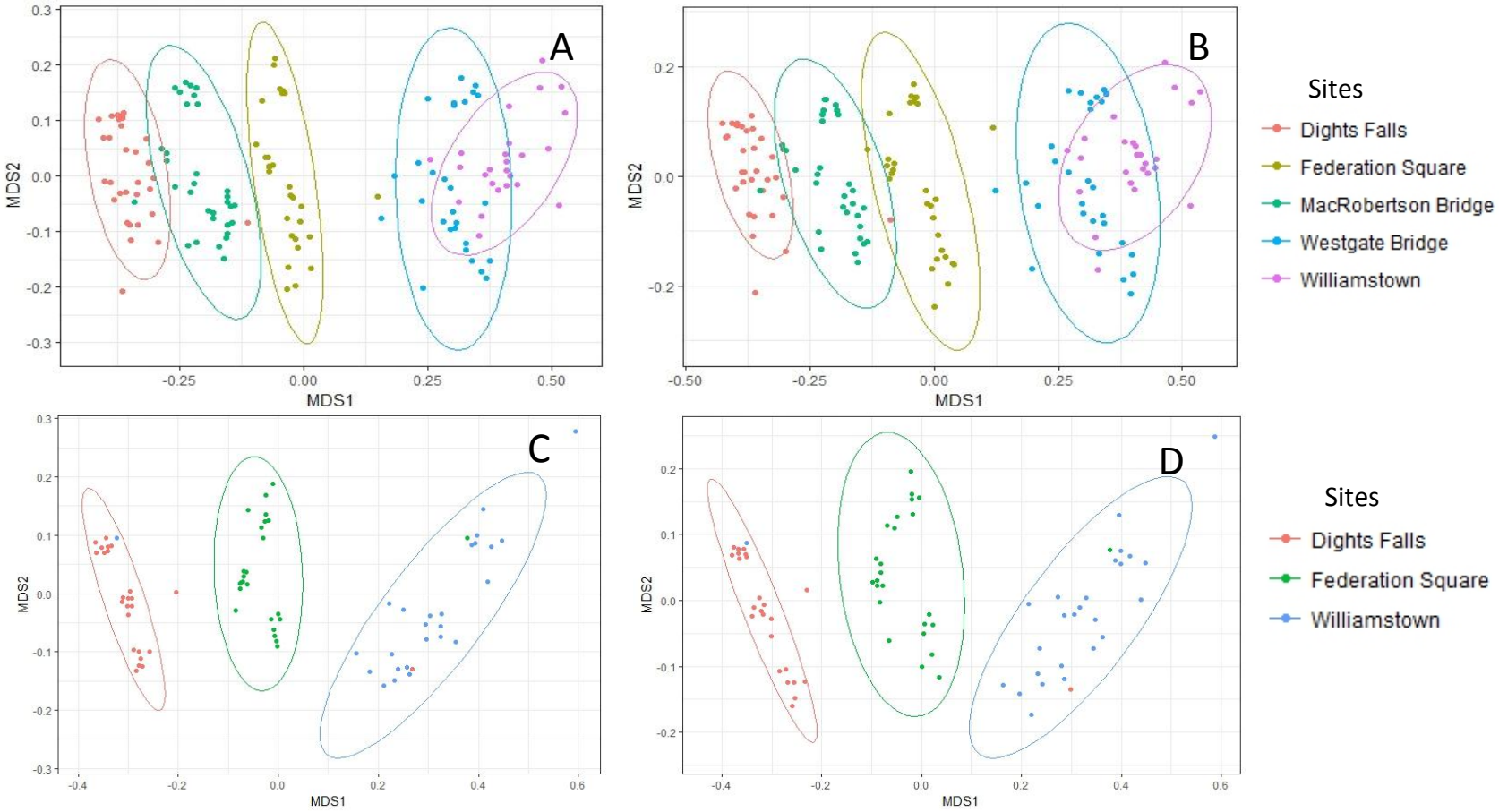


Figure 5.8 Non-metric multidimensional scaling (nMDS) plots of prokaryotic (A and B) and eukaryotic (C and D) biofilm communitiesbased onBray-Curtis dissimilarity matrices (A and C) and Jaccard dissimilarity matrices (B and D) derived from sequencing of rRNA genes. Ellipses represent 95% confidence interval around the centroid. Sites as indicated.

5.3.4 Variation in microbial community structure by site and sampling date

Analysis of Similarities (ANOSIM) was undertaken between all of the biofilm communities aggregated across sample sites, dates and substrate types (Table 5.8) to assess which of these three factors influence community structure using the observed OTU datasets. An R–statistic= 1 occurs when all replicates in a group are more similar to each other than to any other sample. An R-statistic = 0 corresponds to similarities between and within each group being the same. Firstly, ANOSIM results showed that microbial communities were significantly different between sites for both prokaryotic and eukaryotic communities (R = 0.944 and R = 0.916, respectively). Secondly, microbial communities were significantly different between sampling dates for both prokaryotic and eukaryotic communities (R = 0.775 and R = 0.890, respectively). Thirdly, microbial communities were significantly different between coupon substrate types for both prokaryotic and eukaryotic communities coupons (R = 0.396 and R = 0.418, respectively). All R-statistic values were highly significant with a probability of being random of 0.1%. However, the experimental design meant that there was co-variance between the three variables ofsample location, sampling date and coupon type.

Table 5.8 ANOSIM of a Bray-Curtis similarity matrix of prokaryotic and eukaryote biofilm communities for combined factors of sample site, date and coupon type. 999 permutations were performed. P-value <0.001 for all comparisons. n > 60.

Community type	Sample type	R statistic
Prokaryotic	Sites	0.944
	Dates	0.775
	Coupons	0.396
Eukaryotic	Sites	0.916
	Dates	0.890

Coupons	0.418

5.3.5 SIMPER analysis of differences in biofilm microbial communities over time based on Bray-Curtis similarity

Similarity percentage (SIMPER) analysis of the Bray-Curtis similarity index was conducted to assess whether microbial community similarities converged over time. SIMPER analysis was performed using the observed OTU datasets for each coupon type, sampling date and sample site for both the prokaryotic (Table 5.9) and eukaryotic communities (Table 5.10). Having demonstrated that there was very limited variability in community structure between coupons of different substrates for each site and sampling date (see Section 5.3.6), PP, PLA and glass coupon SIMPER percentages were combined for statistical analysis of each sampling date and site. Outliers and normality of the distribution of means were tested and assessed for using box-plots and the Shapiro-Wilk test (see*Appendix III*). Samples did not have outliers and means were normally distributed. Therefore, the parametric statistical test of one-way analysis of variance (One-Way ANOVA) was performed between all sampling dates at each site and between time points Day 7 and Month 3 at each site. The eukaryotic community from Day 7 Dights Falls had only 2 samples. Therefore, the non-parametric statistical tests, Wilcoxon signed ranks test was performed for this group. The prokaryotic community from Day 7 and Month 3 from Williamstown had only 2 samples each. Therefore, these groups were removed from the analysis.

There was no significant difference in similarity between the prokaryotic communities between any of the dates with each other or overall, between Day 7 and Month 3. There was a significant difference between the eukaryotic communities from the combined PP, PLA and glass community similarities at Federation Square, whereby the communities became less similar to each other between Day 7 (72.4% ± 5.7%), and Day 28 (63.7% ± 7.0%) and Month 3 (33.2% ± 5.5%) ($P < 0.05$).

Table 5.9 SIMPER analysis of the prokaryotic biofilm communities based on a Bray-Curtis similarity index. Samples denoted with N/A had ≤2 replicates. One-way ANOVA p-values were generated comparing the means between all four sampling time points and secondly,between Day 7 and Month 3 for the combined biofilm communities at each site.

Coupon Type	**Date**	**DF**	**MRB**	**FS**	**WGB**	**WTN**
Glass	Day 7	51.11	57.86	56.38	62.90	N/A
	Day 14	65.78	69.60	68.73	61.26	68.33
	Day 28	62.41	55.70	72.41	63.65	63.33
	Month 3	61.62	65.83	43.3	63.56	42.52
Polylactic Acid	Day 7	54.76	61.49	65.76	65.65	62.41
	Day 14	41.86	N/A	58.25	N/A	65.68
	Day 28	67.49	47.93	65.48	39.98	49.18
	Month 3	66.45	61.47	60.52	60.16	N/A
Polypropylene	Day 7	61.07	61.1	59.50	60.54	54.62
	Day 14	67.27	62.49	28.0	59.27	62.46
	Day 28	67.27	62.22	66.65	59.53	60.56
	Month 3	67.31	71.04	58.95	66.92	57.78
One-way ANOVA p-value between all dates		0.364	0.110	0.393	0.531	0.204
One-way ANOVA p-value between Day 7 and Month 3		0.05	0.117	0.364	0.843	N/A

DF= Dights Falls, MRB= MacRobertson Bridge, FS= Federation Square, WGB = Westgate Bridge, WTN = Williamstown.

Table 5.10SIMPER analysis of of the eukaryotic biofilm communities based on a Bray-Curtis similarity index. Samples denoted with N/A had ≤2 replicates. One-way ANOVA p-values were generated comparing the means between all four sampling time points and secondly between Day 7 and Month 3 for the combined biofilm communities at each site (significant values are shown in bold). Additionally, Wilcoxon signed rank test p-values were generated comparing the means of Day 7 and Month 3 for communities from Dights Falls.

Coupon Type	**Date**	**DF**	**FS**	**WTN**
Glass	Day 7	76.16	80.86	57.71
	Day 28	79.16	71.30	59.90
	Month 3	69.30	23.02	33.49
Polylactic Acid	Day 7	49.41	74.71	64.13
	Day 28	74.51	68.48	46.14
	Month 3	66.27	41.70	42.00
Polypropylene	Day 7	N/A	61.53	20.91
	Day 28	55.70	51.48	41.33
	Month 3	17.20	34.93	41.51
One-way ANOVA p-value between all dates		0.598	**0.007**	0.683
One-way ANOVA p-value between Day 7 and Month 3		N/A	**0.008**	0.566
Wilcoxon p-value between Day 7 and Month 3		0.655	-	-

DF= Dights Falls, FS= Federation Square, WTN = Williamstown.

5.3.6 Structural similarity of biofilm communities between substrate types

To further evaluate whethermicrobial community structure may be varying with respect to coupon substrate type, permutational multivariate analysis of variance (PERMANOVA) and Monte Carlo tests were performed on the Bray-Curtis similarity matrix for each substrate type and at each site and sampling date using the observed OTU datasets. Therefore the influence of the cofactors of sample site and sampling time point were removed. Results of all relationships that were tested are presented in*Appendix IV*. There was no significant difference (P>0.05) between communities on coupons of different substrate at any individual sample site or sampling date for any of the eukaryotic samples. In the prokaryotic communities, a significant difference between different substrates was noted on only one occasion, with all remaining sites and sampling dates showing no significant differences (P > 0.05) in community structural similarity with respect to substrate type. The site and sampling dates at which a difference in community structural similarity was seen between different substrates was Site 4 (Westgate Bridge at Day 14); between communities on glass (S4D14Glass) and those on PP (S4D14PP); Monte Carlo P = 0.046.

5.3.7 Taxonomic composition of biofilm and water communities

5.3.7.1 Rare biosphere

On average 1,420 ± 45 prokaryotes and 1,649 ± 62 eukaryotes OTUs were identified within the biofilm samples (See Section 5.3.3.1). Figure 5.9 depicts the number of DNA sequence reads per species (≥97% I.D) for both prokaryotic and eukaryotic communities from sample S1L1M3 as an example. The curves display classic rare biosphere profiles as only a few species dominate each community, with the vast majority of species each contributing<0.1% of the DNA sequence reads. In S1L1M3, for the prokaryote community, over 50% of the number of sequence reads were assigned to only 8 species. For the eukaryotic communities,

species composition was even more skewed with over 50% of the number of sequence reads being assigned to only 3 species.

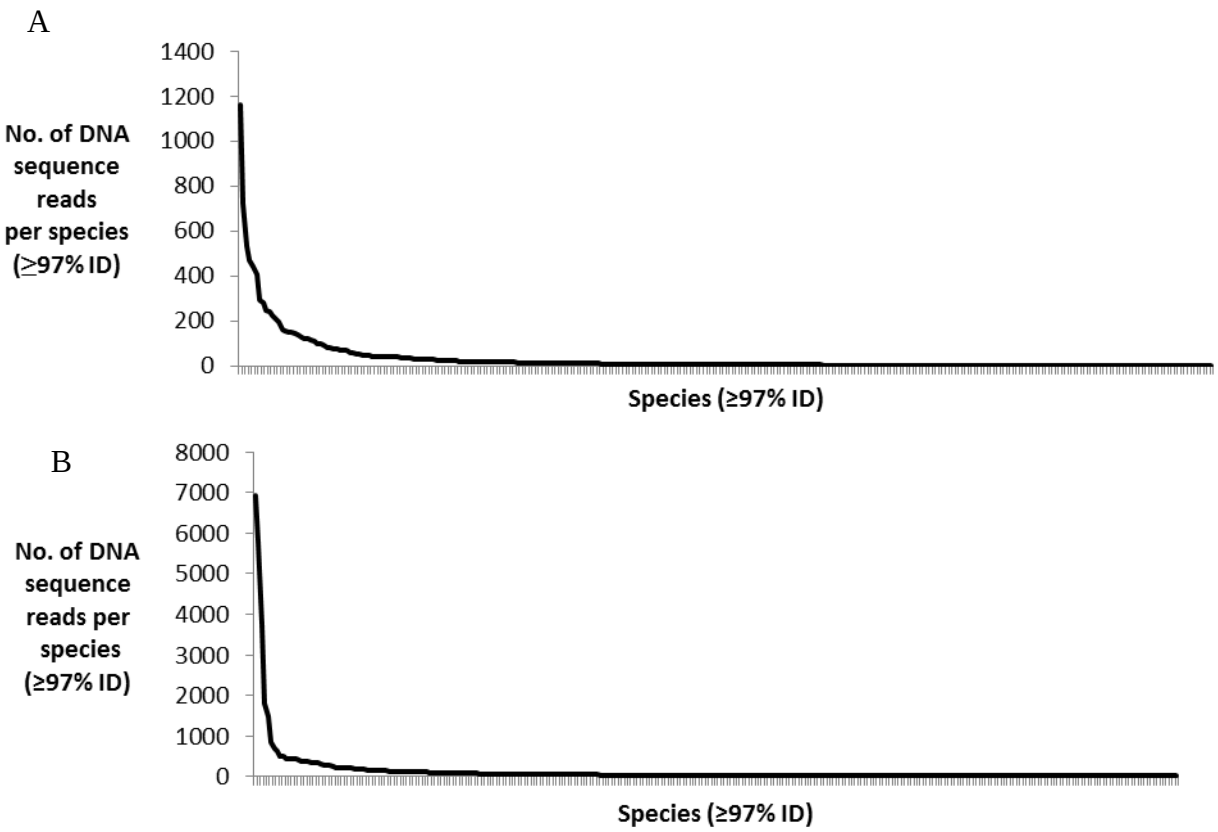


Figure 5.9 Species rank abundance plot of species (≥97% identity) present in sample S1L1M3. A) Prokaryotic community, 302 species present. B) Eukaryote community, 168 species present. Note in both A and B, full sample sets have not been used to shorten the x-axis of the figures.

5.3.7.2 Biofilm and water microbiome taxonomic composition

5.3.7.2.1 Prokaryotic community composition

99.8% of the 16S rRNA gene sequence reads were identified as bacteria, and <0.15% were identified as archaea.. The three most prevalent Proteobacteria classes were alpha- beta- and gamma- proteobacteria. Betaproteobacteria had higher mean relative abundances at Dights

Falls, MacRobertson Bridge and Federation Square in the water communities (37.4% ± 3.8%) and in the biofilm communities (39.6% ± 2.0%) when compared to those in Westgate Bridge and Williamstown communities (4.6% ± 2.6% and 1.5% ± 0.28%, respectively). Alphaproteobacteria and Gammaproteobacteria were more prevalent in the Westgate Bridge and Williamstown communities in both water (Alphaproteobacteria: 22.8% ± 4.1 %; Gammaproteobacteria: 38.6%± 6.7%) and biofilms (Alphaproteobacteria: 42.9% ± 2.3%; Gammaproteobacteria: 20.7% ± 2.7%) when compared to Dights Falls, MacRobertson Bridge and Federation Square in both water (Alphaproteobacteria:1.8% ± 0.4%; Gammaproteobacteria:11.1% ± 3.2%, respectively) and biofilm communities (Alphaproteobacteria:17.8 % ± 1.5%;Gammaproteobacteria:8.0 % ± 1.2%).

Comparison of the relative abundances of the 20 most abundant families from the bacterial biofilm (Figure 5.10) and water (Figure 5.11) communities showed that community structure and composition were primarily associated with habitat type (biofilm vs. water), secondly by spatial (site) differences and thirdly by temporal (sampling date) differences. Amongst the 20 most abundant families, the water and biofilm communities each had seven unique families and shared 13 families in common(Figure 5.12). The seven unique families identified only in the biofilm communities were: Oceanospirillaceae, Erythrobacteraceae, Arenicellaceae, Methylococcaceae, Enterobacteriaceae, Granulosicoccaceae and Zoogloeaceae. The seven unique families identified only in the water communities were: Pelagibacteraceae, Microbacteriaceae, Chitinophagaceae, Methylophilaceae, Yersiniaceae, Vibrionaceae and Chromobacteriaceae. The 13 shared families were: Rhodobacteraceae, Comamonadaceae, Flavobacteriaceae, Colwelliaceae, Moraxellaceae, Sphingomonadaceae, Verrucomicrobiaceae, Methylophilaceae, Alteromonadaceae, Pseudomonadaceae, Burkholderiaceae, Hyphomonadaceae and Campylobacteraceae. No archaeal families were identified among the 20 most abundant prokaryote families. It was noted that these 13 shared

bacterial families differed in their relative abundance between the biofilm and water communities. For example, Burkholderiaceae had a mean relative abundance of 7.2% ± 1.5 % in the water communities and was present at all five sites but had a mean relative abundance of >0.5 % only in the biofilm communities at Dights Falls. Conversely, Rhodobacteraceae had a mean relative abundance of 23% ± 1.6% in the biofilm communities, and a lower mean relative abundance of 3.6% ± 1.0% in the water communities.

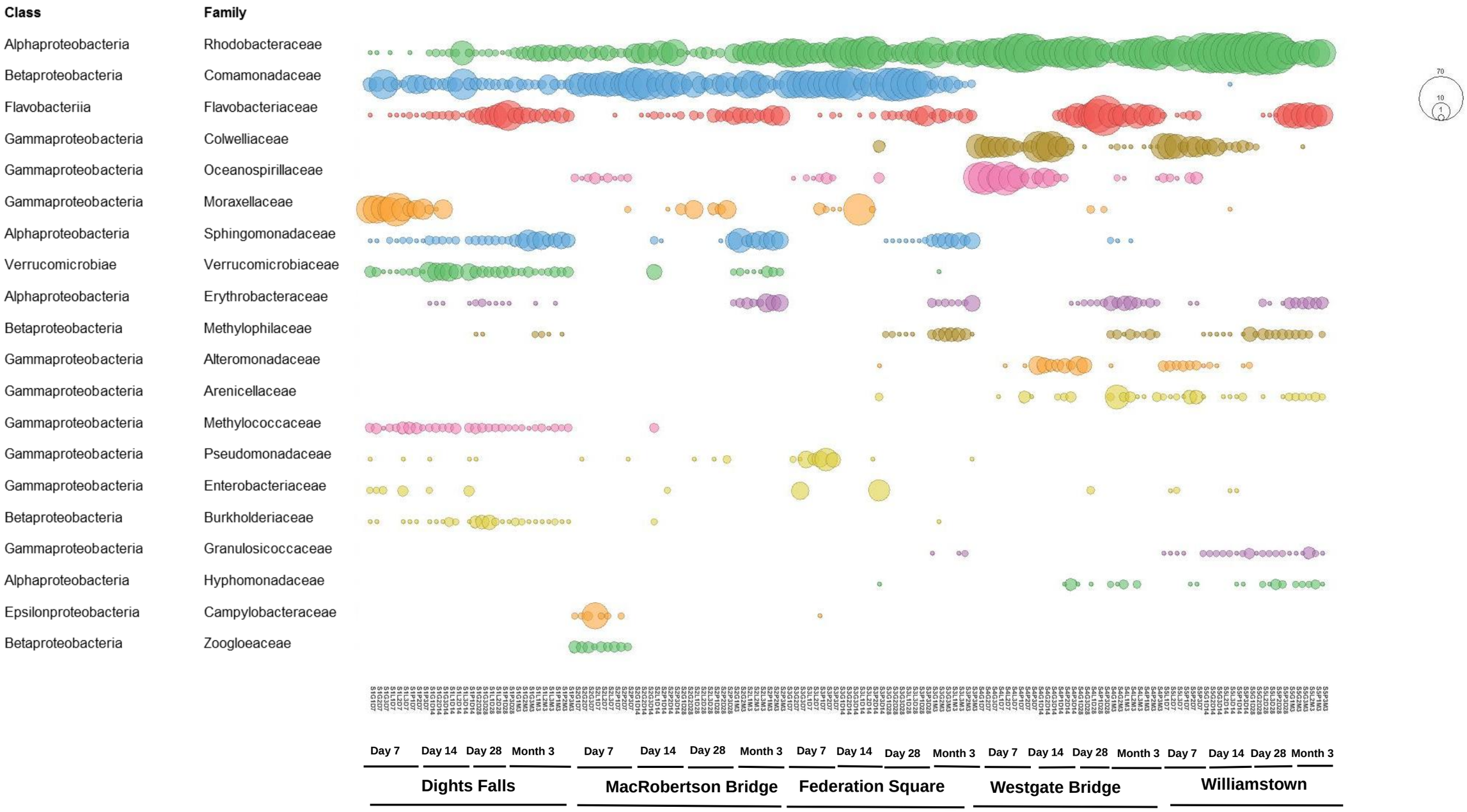


Figure 5.10 Proportions of the bacterial families with the 20 highest relative abundances within biofilm communities. Bacterial families grouped by sampling site and with than by sampling dates of Day 7, Day 14, Day 28 and Month 3 (based on 16S rRNA gene analysis). Legend indicates proportions of sequence reads per family. Software package Megan6 was used to generate figure.

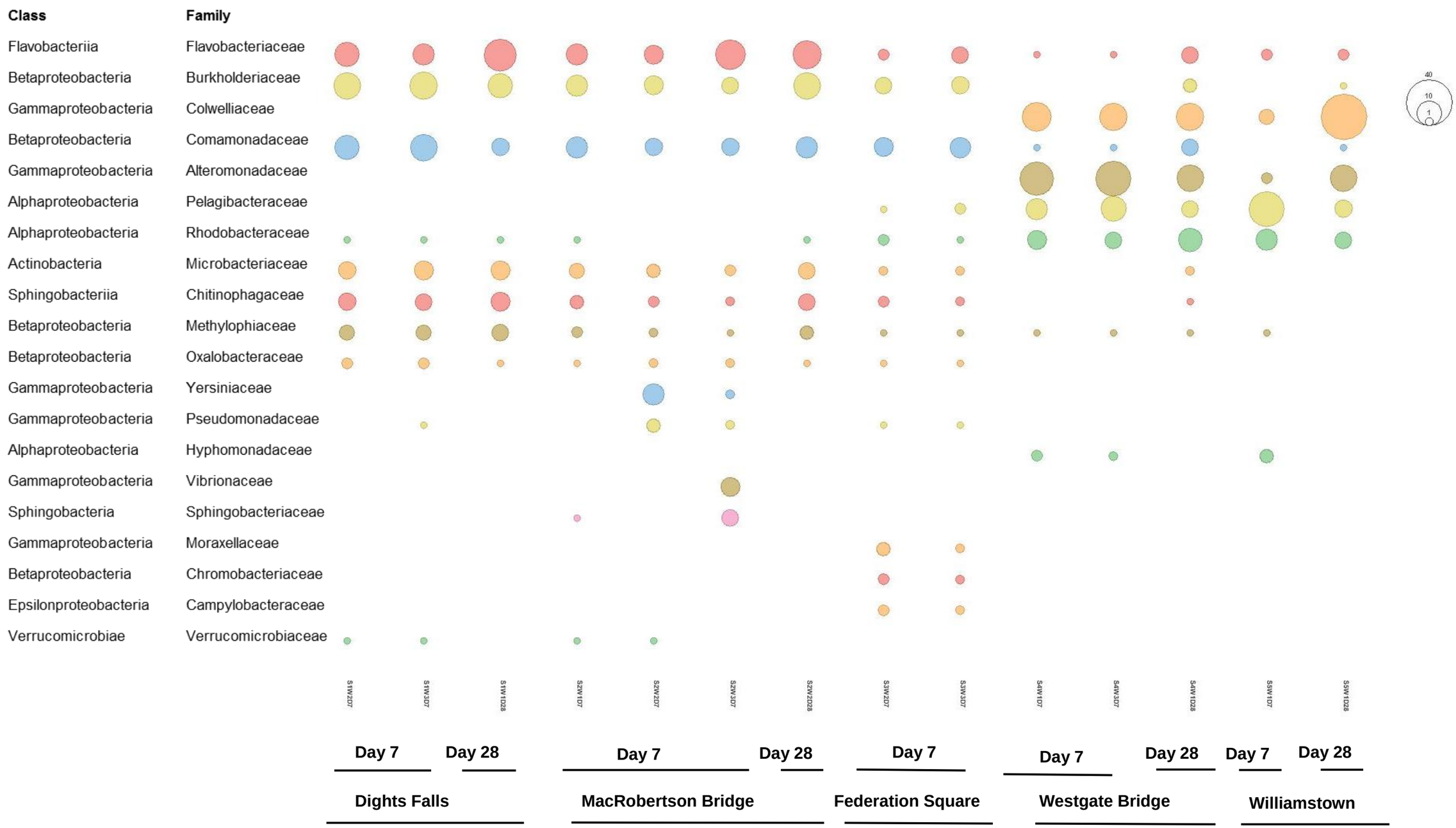


Figure 5.11 Proportions of the bacterial families with the20 highest relative abundances within water communities. Bacterial families grouped by sampling site and by sampling dates of Day 7, Day 14, Day 28 and Month 3 (based on 16S rRNA gene analysis). Circle area indicates proportions of sequence reads per family (see scale). Software package Megan6 was used to generate figure.

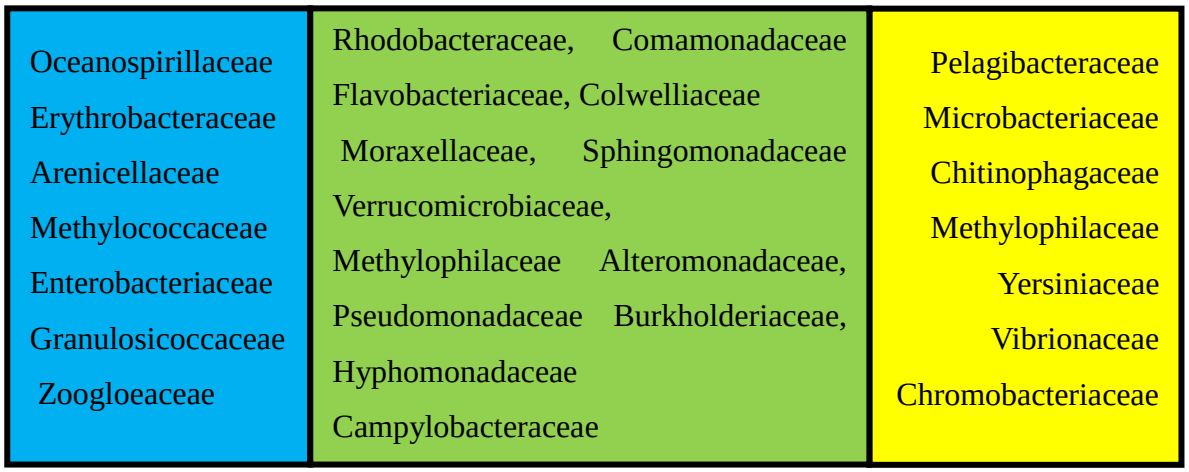


Figure 5.12Venn diagram of common prokaryotic families present in biofilm and water communities. Blue) Families specific to biofilm communities,Yellow)Families specific to watercommunities,Green) Families present in both biofilm and water communities.

In the biofilm communities, the distribution of particular bacterial families varied between sites. For example; Comamonadaceae and Sphingomonadaceae had higher relative abundance in the Dights Falls, MacRobertson Bridge and Federation Square communities (18.1% ± 1.3% and 3.6% ± 0.5%,respectively) compared to the Westgate Bridge and Williamstown communities (Comamonadaceae: 1.5% ± 0.5%; Sphingomonadaceae: 0.8% ± 0.3%). Whilst Verrucomicrobiaceae had a higher relative abundance in the Dights Falls communities and in the least saline estuarine site of MacRobertson Bridge (average EC = 5,344 $\mu S/cm^2$± 805$\mu S/cm^2$) communities (3.1% ± 0.5%) when compared to the other three downstream sites (<0.5%). Conversely, Colwelliaceae, Alteromonadaceae, Arenicellaceae, Granulosicoccaceae and Hyphomonadaceae had higher relative abundances in the Westgate Bridge and Williamstown communities (Colwelliaceae: 8.7% ± 1.5%; Alteromonadaceae: 2.1% ± 0.5%; Arenicellaceae: 2.1% ± 0.5%; Granulosicoccaceae: 0.8% ± 0.2%;

Hyphomonadaceae: 0.8% ± 0.2%) than in the communities at the other three upstream sites (<0.5%).

In the water communities, families that had higher relative abundances at some sites were: Flavobacteriaceae, Burkholderiaceae, Comamonadaceae and Microbacteriaceae in the Dights Falls, MacRobertson Bridge and Federation Square communities (Flavobacteriaceae: 12% ± 2.0%; Burkholderiaceae: 10.6% ± 1.3%; Comamonadaceae: 9.6% ± 0.9%; Microbacteriaceae: 5.0% ± 0.8%) compared to the Westgate Bridge and Williamstown communities (Flavobacteriaceae: 2.8% ± 0.8%; Burkholderiaceae: 1.0% ± 0.7 %; Comamonadaceae: 1.8% ± 1.0%; Microbacteriaceae: 0.4% ± 0.35%). Conversely, Alteromonadaceae, Colwelliaceae and Pelagibacteraceae had higher relative abundances in the Westgate Bridge and Williamstown communities (Alteromonadaceae: 19.8% ± 5.8%; Colwelliaceae: 16.6% ± 3.7%; Pelagibacteraceae: 12.6% ± 3.2%) when compared to the other three upstream sites (<0.5%).

Temporal changes in the biofilm communities were observed (Figure 5.10). In the biofilm communities, at all sites, Flavobacteriaceae increased in relative abundance between Day 14 (1.1% ± 0.3%) and Day 28 (12.6% ± 1.4%). Sphingomonadaceae relative abundance increased at the Dights Fall and MacRobertson Bridge and the Federation Squares site between Day 7 (2.3% ± 0.3%) and Month 3 (10.6% ± 1.3%). Erythrobacteraceae were initially present at low relative abundance (<0.5%), in the MacRobertson Bridge, Federation Square, Westgate Bridge and Williamstown communities before increasing to a relative abundance of 5.6% ± 0.6% between Day 28 and Month 3. Methylophilaceae also increased in relative abundance at the Federation Square, Westgate Bridge and Williamstown sites from <0.5% between Day 7 and 14 to 3.1% ± 0.4% by Month 3.

In the biofilm communities, three families markedly decreased in their relative abundance between sampling dates; they were Colwelliaceae, Oceanospirillaceae andMoraxellaceae.

Colwelliaceae decreased in relative abundance at the Westgate Bridge and Williamstown sites from 15.8% ± 2.0% and 13.4% ± 0.2%, respectively between Day 7 and Day 14 to <0.5% by Month 3. Oceanospirillaceae had higher relative abundances at Day 7 and Day 14 (7.4% ±1.4%) in the MacRobertson Bridge, Federation Square, Westgate Bridge and Williamstown communities when compared to Day 28 and Month 3 when they comprised <0.5% of the communities. Moraxellaceae also decreased in relative abundance at Dights Falls, from 14.9% ± 3.4% between Day 7 and Day 28 to <0.5% between Day 28 and Month 3.Campylobacteraceae and Zoogloeaceae only occurred once with a relative abundance of >0.5% at MacRobertson Bridge at Day 7. Temporal variation in the composition of the water bacterial communities could not be considered due to uneven numbers of samples from the two sampling dates (10 samples for Day 7 and 4 samples for Day 28).

5.3.7.2.2 Eukaryotic community composition

In the eukaryotic communities, Bacillariophyta (diatoms) was the most prevalent phylum in both water (13.4% ± 2.5%) and biofilms (28.4% ± 2.7%) (data not shown). Chlorophyta (green algae) was the second most prevalent phylum in the water communities (10.7% ± 3.9 %), whilst Arthropoda (arthropods) was the second most prevalent phylum in the biofilm communities (6.8% ± 2.3%). The relative abundance of phyla varied amongst some sites. For example, in both the water and biofilm communities, Bacillariophyta were more prevalent at the Dights Fall and Federation Square sites (19.2% ± 2.6% and 40.3% ± 2.9% respectively) compared to the Williamstown site (2.8% ± 0.9% and 6.1% ± 1.8% respectively). Conversely, Chlorophyta were more prevalent in the water communities at the Williamstown site (29.5% ± 5.4%) compared to the Dights Falls and Federation Square sites (<0.5%). Arthropoda were also more prevalent in the biofilm communities at the Williamstown site

(19.0% ± 5.8%) when compared to the communities at Dights Falls and Federation Square sites (<0.5%).

The overall patterns observed for the composition of families in the eukaryotic communities were the same as those observed for the bacterial communities, in that the primary differences were between either the sample being a biofilm (Figure 5.13) or a water sample (Figure 5.14) and secondly due to spatial and temporal factors. Amongst the 20 most abundant families, the water and biofilm communities had 15 unique families each and shared five families in common (Figure 5.15). The 15 unique families in the biofilm communities were: Acinetidae (ciliates), Zoothamniidae (ciliates), Gomphonemataceae (diatoms), Harpacticidae (crustaceans), Ectocarpaceae (brown algae), Misophriidae (crustaceans), Surirellaceae (diatoms), Aphanochaetaceae (green algae), Pseudocharaciopsidaceae (microalgae), Laminariaceae (brown algae), Tribonemataceae (yellow-green algae), Pteriidae (molluscs), Bodonidae (protozoa), Chroomonadaceae (green algae) and Ephelotidae (ciliates). The 15 unique families in thewater communities were: Mallomonadaceae (golden algae), Bathycoccaceae (green algae), Stentoridae (ciliates), Mamiellaceae (green algae), Mytilidae (molluscs), Strobilidiidae (ciliates), Balanidae (crustaceans), Thalassiosiraceae (diatoms), Adeleidae (protozoa), Asteriidae (sea star), Tontoniidae (protozoa), Holostichidae (ciliate), Ancyromonadidae (protozoa), Geminigeraceae (microalgae) and Chlorellaceae (green algae). The five shared families were: Melosiraceae (diatoms), Vorticellidae (ciliate), Naviculaceae (diatoms), Amphileptidae (ciliates) and Chaetophoraceae (diatoms).

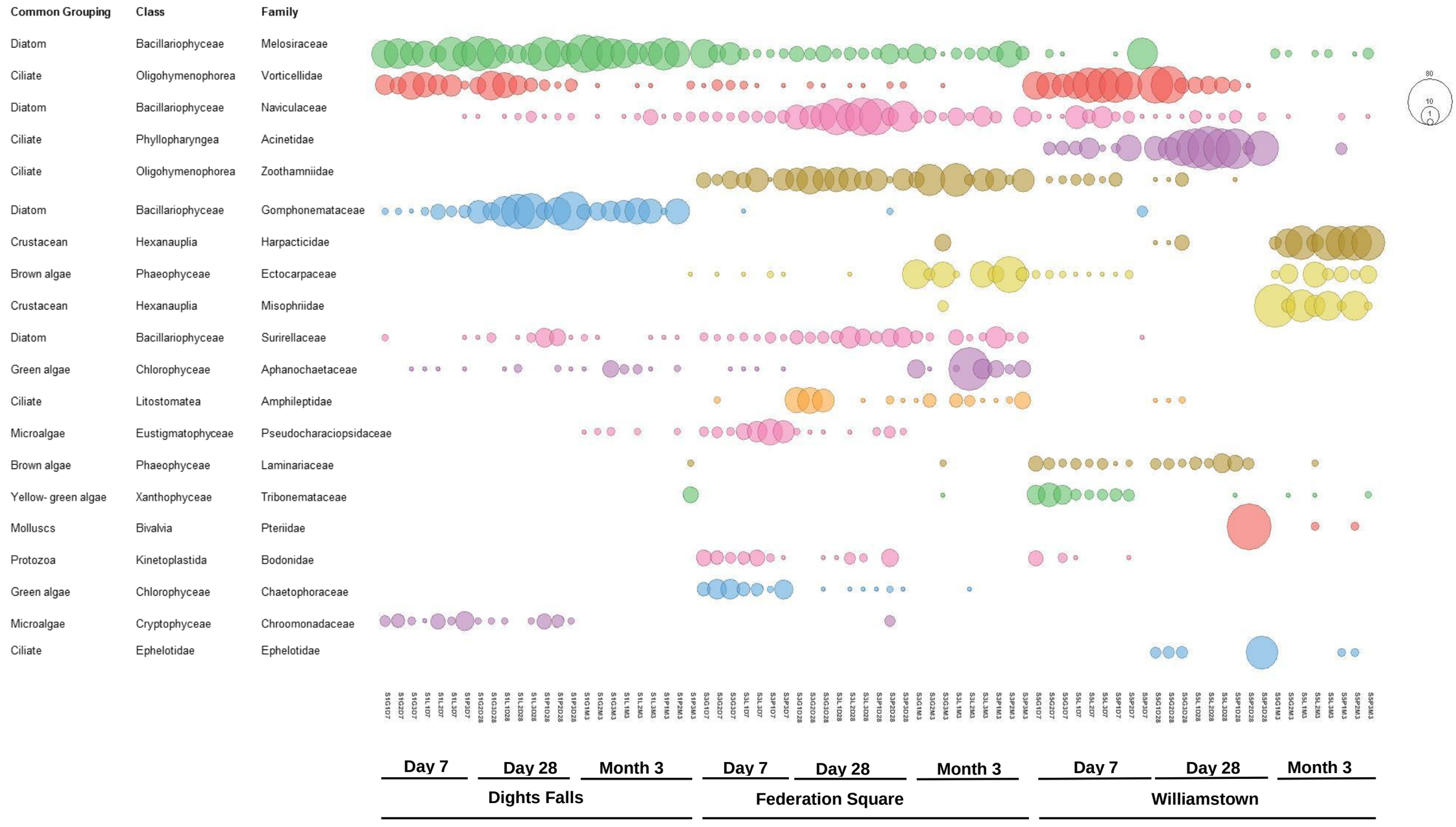


Figure 5.13 Proportions of the eukaryotic families with the20 highest relative abundances within biofilm communities. Eukaryotic families grouped by sampling site and by sampling dates of Day 7, Day 14, Day 28 and Month 3 (based on 18S rRNA gene analysis). Circle area indicates proportions of sequence reads per family (see scale). Software package Megan6 was used to generate figure.

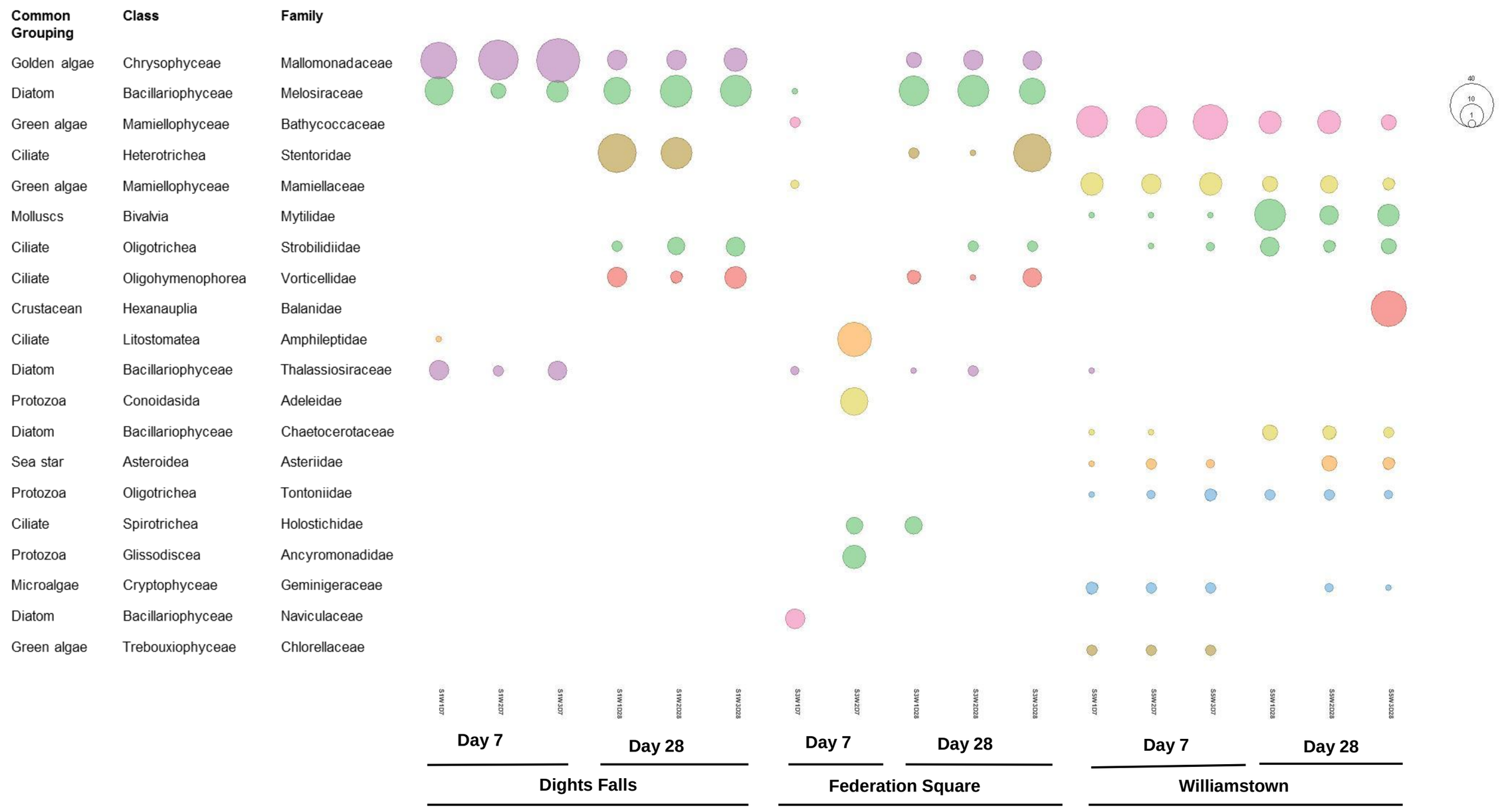


Figure 5.14 Proportions of the eukaryotic families with the 20 highest relative abundances within water communities. Eukaryotic families grouped by sampling site and with than by sampling dates of Day 7, Day 14, Day 28 and Month 3 (based on 18S rRNA gene analysis). Legend indicates proportions of sequence reads per family. Software package Megan6 was used to generate figure.

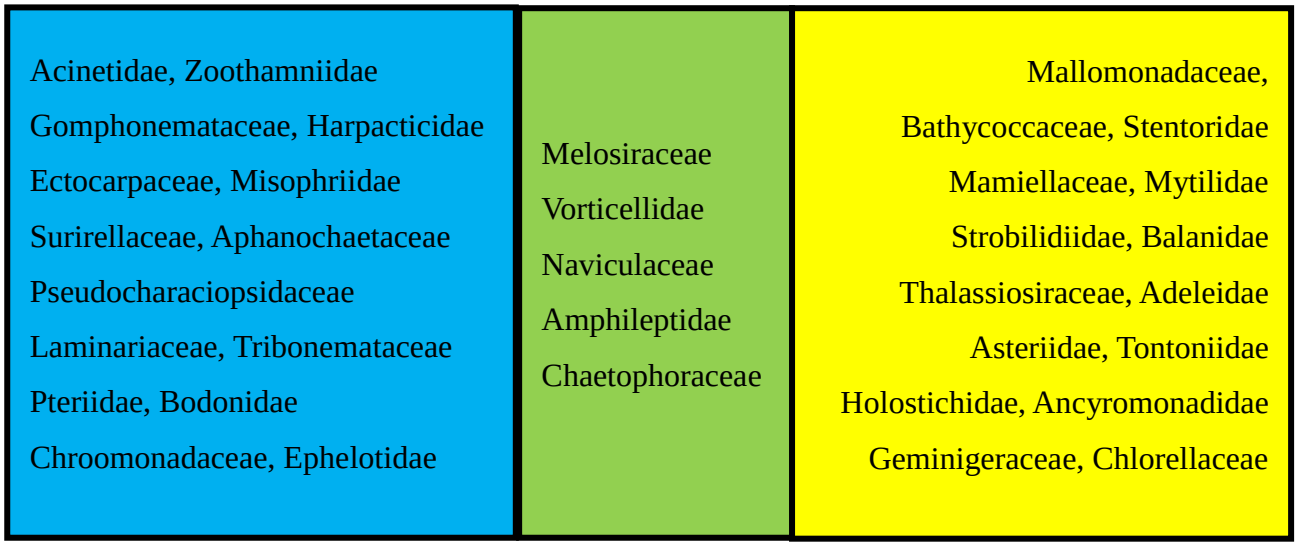


Figure 5.15Venn diagram of common eukaryotic families present in biofilm and water communities. Blue) Families specific to biofilm communities,Yellow)Families specific to watercommunities,Green) Families present in both biofilm and water communities.

In the biofilm communities, families that were more prevalent at some sites than others were the diatoms Melosiraceae, Gomphonemataceae and the microalgae Chroomonadaceae in the Dights Falls communities (Melosiraceae: 28.2% ± 2.7%; Gomphonemataceae: 17.6% ± 3.2 % and Chroomonadaceae: 2.9% ± 0.8%) when compared to the Federation Square and Williamstown communities (Federation Square and Williamstown combined: Melosiraceae: 5.7% ± 1.1%; Gomphonemataceae: <0.5% and Chroomonadaceae: <0.5%). When compared to the bacterial biofilm communities, the eukaryotic biofilm estuarine (Federation Square) communities were more distinct from those in both the freshwater (Dights Falls) and marine (Williamstown) communities. Naviculaceae, Zoothamniidae, Surirellaceae, Amphileptidae, Bodonidae and Chaetophoraceae were more abundant at Federation Square (Naviculaceae: 16.3% ± 3.2%; Zoothamniidae: 16.0% ± 2.1%; Surirellaceae: 6.3% ± 1.0%; Amphileptidae: 4.6% ± 1.5%; Bodonidae: 2.7% ± 0.8%; Chaetophoraceae: 3.2% ± 1.0%) than in either Dights Falls or Williamstown communities (< 2.5%). Acinetidae, Harpacticidae, Misophriidae, Laminariaceae and Tribonemataceae were more prevalent in the Williamstown

communities (Acinetidae: 18.2% ± 4.5%; Harpacticidae: 10.9% ± 3.5%; Misophriidae: 8.1% ± 3.3%; Laminariaceae: 3.5% ± 0.7%: Tribonemataceae: 3.2% ± 1.1 %) than in either Dights Falls or Federation Square communities (<0.5%). Interestingly, between Day 7 and Day 28, Vorticellidae had a greater relative abundance in both the Dights Falls (14.8% ± 2.4%) and Williamstown (23.7% ± 4.3%) communities when compared to the Federation Square communities (1.5% ± 0.3%).

Temporal changes in the eukaryotic biofilm communities were observed (Figure 5.13). In both the Federation Square and Williamstown communities, Ectocarpaceae had low relative abundance between Day 7 and Day 28 (0.6% ± 0.7%) and increased in relative abundance by Month 3 (13.8% ± 3.2%). Harpacticidae and Misophriidae had higher relative abundances in the Williamstown communities at Month 3 (34% ± 5.2% and 26.3% ± 7.6% respectively), having relative abundances of<0.5% in all other sampling dates. In the Dights Falls and Williamstown communities, Vorticellidae decreased in relative abundance from Day 7 and Day 28 (14.8% ± 2.4% and 23.7% ± 4.3%, respectively) compared to < 0.5% by Month 3. In the Federation Square communities, Pseudocharaciopsidaceae, Chaetophoraceae and Bodonidae decreased in relative abundance from Day 7 (12.0% ± 3.1 %, 10.2% ± 2.0% and 6.3% ± 1.2%, respectively) to Day 28 and Month 3 (0.8% ± 0.4 %, <0.5 % and 1.3% ± 0.7 %, respectively). Interestingly, two families (Naviculaceae and Gomphonemataceae) increased in relative abundance between Day 7 and Day 28, and then decreased in relative abundance between Day 28 and Month 3. In the Federation Square communities, Naviculaceae increased in relative abundance from Day 7 (4.8% ± 0.5%) to Day 28 (33.9% ± 4.8%) after which they decreased in relative abundance by Month 3 (7.6% ± 1.8%). Whilst in the Dights Falls communities, Gomphonemataceae increased in relative abundance from Day 7 (4.1% ± 1.0%) to Day 28 (32.4% ± 5.7%) after which they the decreased in relative abundance (14.8% ± 3.1%).

In the water communities, families that were more prevalent at some sites were; Mallomonadaceae, Melosiraceae and Stentoridae in the Dights Falls and Federation Square communities (Mallomonadaceae: 16.3% ± 4.6%; Melosiraceae: 15.7% ± 2.8%; Stentoridae: 9.3% ± 4.4%) than in the Williamstown communities (<0.5%) and; Bathycoccaceae, Mamiellaceae and Mytilidae in the Williamstown communities (Bathycoccaceae: 19.0% ± 3.5%; Mamiellaceae: 9.0% ± 1.4%; Mytilidae: 8.2% ± 3.5%) when compared to the Dights Falls and Federation Square communities (<0.5%). Temporal changes also occurred in the water communities. For example, Stentoridae, Strobilidiidae and Vorticellidae had greater relative abundances at Day 28 (Stentoridae: 11.3% ± 5.1%; Strobilidiidae: 5.0% ± 1.0%; Vorticellidae: 4.6% ± 1.5%) than in the Day 7 communities (<0.05%). Mytilidae became more abundant in the Williamstown communities by Day 28 (15.3% ± 4.0%) when compared to Day 7 (1.0%).

5.3.8 Putative plastic degrading microorganisms and pathogens in biofilm and water communities

5.3.8.1 Plastic degrading organisms

Taxa identified during this study were compared to a list of 43 bacterial and 32 fungal species that have been identified in literature (for full list of taxa see Section1.5.2) to degrade various types of plastics under laboratory conditions. Of these 75 organisms, only one bacterium and one fungus were identified in this current study(Table 5.11). The bacterium*Bordetella petrii* was identified in 94 % of samples, and was more frequently detected in the water communities when compared to the biofilm communities (100 % and 87 %, respectively); the mean number of 16S rRNA gene amplicon reads from this species was also higher in the water communities when compared to the biofilm communities (70.1 ± 20.9 and 9.0 ± 1.5, respectively).The highest relative abundance of *B. petrii* DNA sequence reads from biofilm communities was in sample S3L1M3 (0.23%) and the highestrelative abundance of *B.*

*petrii*DNA sequence reads from water communities was in sample S1W2D7 (0.39%).The fungus *Aspergillusfumigatus*was identified in 58 % of samples, and was more frequently detected in the water communities when compared to the biofilm communities (75 % and 40 %, respectively); the mean number of 18S rRNA gene amplicon reads from this species was also higher in the water communities when compared to the biofilm communities (210 ± 58, and 2.7 ± 0.6, respectively). More than 98% of these reads were from the Williamstown site. The highest relative abundance of *A. fumigatus* DNA sequence reads from biofilm communities was in sample S5P3D28 (0.08%) and the highest relative abundance proportion of *A. fumigatus* DNA sequence reads from water communities was in sample S5W3D7 (2.7%).

Table 5.11 Presence of taxa identified that have been reported to possess plastic degrading capabilities from 16S rRNA gene and 18S rRNA gene sequencing.*

		Total community		**Biofilm community**		**Water community**	
Target gene	**Organism**	**Present (%)**	**Number of sequence reads**	**Present (%)**	**Number of sequence reads**	**Present (%)**	**Number of sequence reads**
16S rRNA	*Bordetella petrii*	94	2,592	87	1440	100	1,152
18S rRNA	*Aspergillus fumigatus*	58	3,932	40	151	75	3,781

*Sample sizes for16S rRNA gene sequence samples for the biofilm and water communities were 150 and 16, respectively. Sample sizes for the 18S rRNA gene sequence samples for the biofilm and water communities were 73 and 20, respectively.

5.3.8.2 Pathogenic bacteria and harmful algae

16S rRNA gene sequences from biofilm and water samples were screened against a comprehensive list of 758 pathogenic bacteria (BG Chemie, 1992)(see *Appendix V*). 16S rRNA genes related to those from a total of 31 pathogens were identified (Table 5.12), with

all 31 detected within the biofilms communities and 24 detected within the water communities. The three most frequently detected 16S rRNA genes from pathogens in the biofilm samples were from *Pseudomonas anguilliseptica*, *Acinetobacter johnsonii* and *A. lwoffii* being detected in 133, 122 and 113 samples, respectively out of 150. For the water samples *P. anguilliseptica*, *A. johnsonii* and *A. lwoffii* 16S rRNA genes were detected in 11, 12 and 10 samples out of 14, respectively. The relative abundance of the pathogen DNA sequence reads were generally low, representing<1% of the total reads in all samples except for *P. anguilliseptica*16S rRNA genes which comprised 11.6% of all reads in S3P2D7.

Table 5.12 Distribution and relative abundance of potential pathogenic bacteria in biofilm and water communities. Total number of samples in which potential pathogen 16S rRNA gene sequences were present, total number of 16S rRNA gene sequence reads per pathogen and the highest relative abundance of pathogen 16S rRNA gene sequence reads from a single sample are presented. N/D indicates pathogen 16S rRNA genes that were not detected in water.

	Biofilm			Water		
Pathogenic Species	**Number of samples present ***	**Total number of reads**	**Highest relative abundance (%)**	**Number of samples present #**	**Total number of reads**	**Highest relative abundance (%)**
Pseudomonas anguilliseptica	133	19,197	11.6	10	173	0.1
Acinetobacter johnsonii	122	3,114	0.5	12	552	0.5
Flavobacterium psychrophilum (Flexibacter pyschrophilus)	85	2,799	1.0	11	75	0.03
Acinetobacter lwoffii	113	1,450	0.2	10	351	0.3
Stenotrophomonas maltophilia	71	830	0.3	9	147	0.09
Staphylococcus aureus	67	775	0.1	12	48	0.02
Bacillus cereus	50	211	0.04	10	37	0.02
Acinetobacter calcoaceticus	48	298	0.09	6	186	0.2
Steptococcus gallolyticus	32	180	0.06	10	55	0.03
Rickettsia conorii	43	179	0.04	2	4	<0.01
Legionella longbeachae	49	142	0.01	9	39	0.02
Clostridium perfringens	23	58	0.01	6	60	0.1
Clostridium difficile	34	99	0.02	5	16	0.01
Fusibacterium mortiferum	15	32	0.02	10	47	0.03
Steptococcus salivarius	21	71	0.06	3	5	0.01

Acidaminococcus fermentans	16	56	0.02	3	7	0.02
Neisseria weaveri	7	55	0.1	1	2	<0.01
Fusibacterium periodonticum	2	54	0.2	0	0	N/D
Listonella anguillarum	13	42	0.02	4	9	0.01
Sanguibacter inulinus	21	42	0.01	3	6	<0.01
Mycoplasma verecundum	2	14	0.02	1	6	0.01
Clostridium magnum	2	8	0.01	4	20	0.02
Pseudomonas aeruginosa	8	25	0.02	0	0	N/D
Fusibacterium nucleatum	5	22	0.02	0	0	N/D
Photobacterium damsela (Listonella damsela)	9	20	0.01	1	2	<0.01
Propionibacterium acnes	8	16	0.004	1	5	0.01
Rickettsia sibirica	2	11	0.01	0	0	N/D
Porphyromonas catoniae (Oribaculum catoniae)	2	6	0.01	0	0	N/D
Camplyobacter jejuni	1	1	0.002	1	4	<0.01
Prevotella buccalis (Bacteroides buccalis)	1	5	0.004	0	0	N/D
Mycoplasma phocacerebrale	1	3	0.01	0	0	N/D

*Out of 150 samples

[#] Out of 14 samples

18S rRNA gene biofilm and water samples were screened against a comprehensive list of 263 harmful algae (United Nations Environment Programme, 2018b) (see*Appendix VI*). A total of nine harmful algae (HA) were identified on the basis of 18S rRNA gene sequencing (Table 5.13). All nine HA were identified in the biofilm communities and seven were identified in the water communities. The three most frequently detected HA in the biofilms were *Pseudo-nitzchia australis, Gonyaulax spinifera* and *Amphora coffeaeformis* being detected in 45, 27 and 19 samples out of 95, respectively. The three most frequently detected HA in the water samples were *P. australis, P. pungens* and *Aureococcus anophagefferens* being detected in 20, 6 and 4 samples out of 25, respectively. The relative abundance of the HA 18S rRNA gene sequence reads were low in both biofilm and water communities, representing ≤0.07% of the total reads in all samples.

Table 5.13 Presence of harmful algae in the biofilm and water communities. Total number of samples in which 18S rRNA genes from harmful algae that were present, total amount of DNA sequence reads per harmful algae and the highest relative abundance of harmful algae DNA sequence reads from a single sample. N/D indicates species that were not detected in water.

		Biofilm			Water		
Common grouping	**Harmful species**	**Total number of reads**	**Number of samples present***	**Highest relative abundance (%)**	**Total number of reads**	**Number of samples present#**	**Highest relative abundance (%)**
Diatom	*Amphora coffeaeformis*	162	19	0.06	14	3	0.01
Diatom	*Pseudo-nitzchia australis*	247	45	0.06	280	20	0.1
Dinoflagellate	*Gonyaulax spinifera*	135	27	0.06	1	1	0.001
Ochrophyta	*Aureococcus anophagefferens*	99	5	0.07	8	4	0.01
Diatom	*Pseudo-nitzchia pungens*	4	3	0.003	31	6	0.02
Diatom	*Pseudo-nitzchia turgidula*	23	10	0.01	0	0	N/D
Dinoflagellate	*Pfiesteria piscicida*	21	9	0.01	0	0	N/D
Dinoflagellate	*Karenia mikimotoi*	3	1	0.003	9	2	0.01
Diatom	*Nitzschia actydrophila*	8	5	0.003	2	2	0.003

*Out of 95 samples

#Out of 25 samples

5.3.9 Negative controls

Prior to DNA sequencing, the concentration of PCR products were quantified using a Qubit fluorometer after the Illumina® barcoded primers had been attached to the amplicons. The 16S rRNA gene samples had a mean concentration of 10.7 ng μL^{-1} ± 0.4 ng μL^{-1} of DNA forthe coupon and water samples, and 3.7 ng μL^{-1} ± 1.0 ng μL^{-1} for the extraction blank amplicons. The 18S rRNA gene samples had a mean concentration of 28.6 ng μL^{-1} ± 1.0 ng μL^{-1} of DNA for the coupon and water samples, and 3.4 ng μL^{-1} ± 1.2 ng μL^{-1} for the extraction blank amplicons. Prior to sequencing, samples were pooled to a concentration of 40 nM mL^{-1} before continuing with the preparation for sequencing(see Section 5.2.2).

The potential influence of contaminant DNA upon the interpretation of the sequence data was assessed. As indicated above, the concentrations of the DNA in the amplicons of the blank extracts were initially an order of magnitude lower than that of the samples. However, during the pooling process the amount of DNA from each sample to be sequenced was normalised, increasing the relative proportion of DNA from the extraction blanks within the pool used for sequencing. Bootstrapped non-metric multidimensional scaling (nMDS) of the Bray-Curtis similarities revealed that the communities from the extraction blanks were completely distinct from the biofilm and water communities when grouped by sample date, sample site or sample type for both 16S rRNA gene sequences (Figure 5.16) and 18S rRNA gene sequences (Figure 5.17). Therefore, the presence of these potential contaminant sequences in the samples had minimal effect on the interpretation of the sequence data for the analysis of the biofilm and water communities.

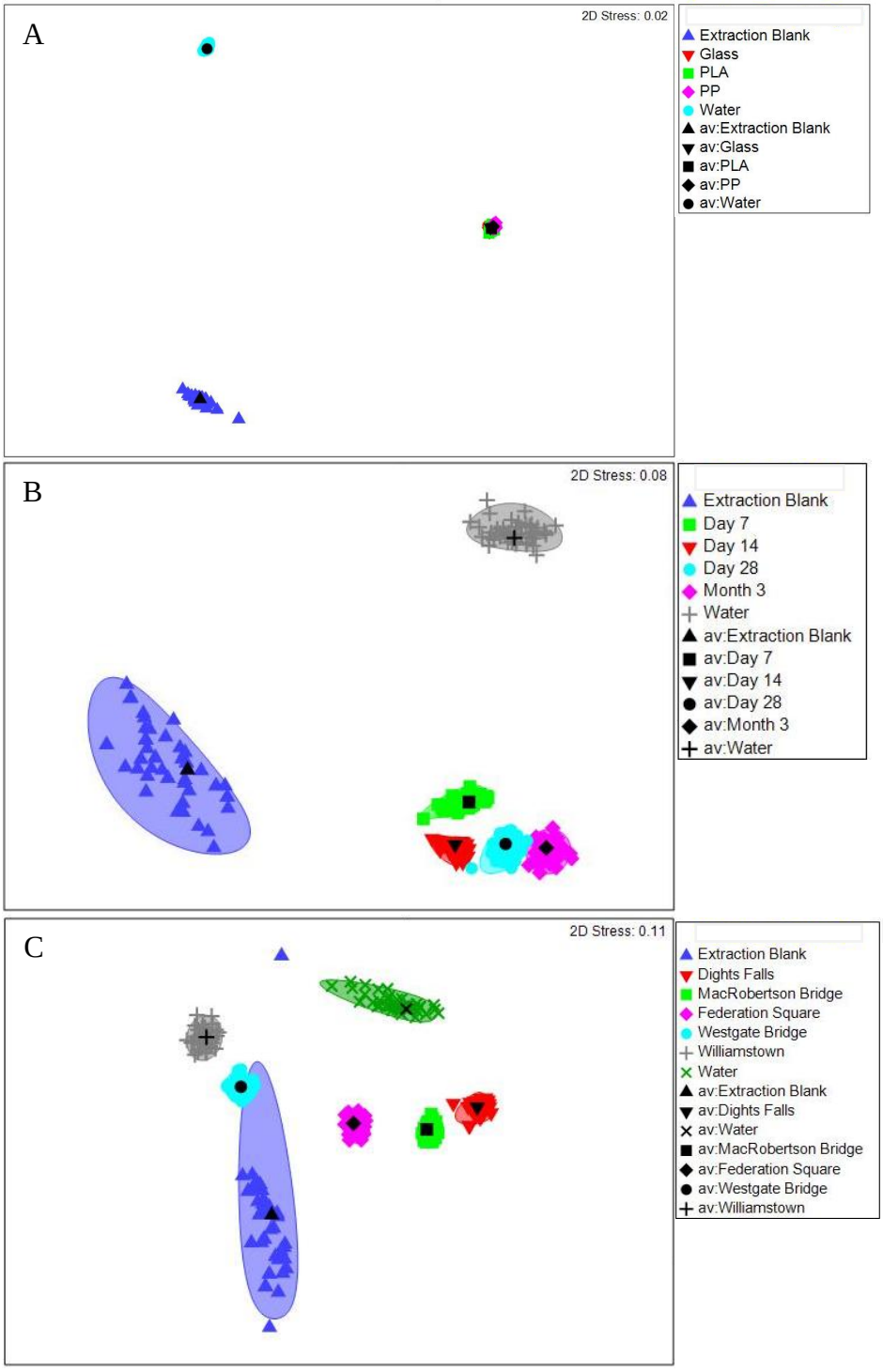


Figure 5.16 Non-metric multidimensional scaling (nMDS) representation of 75 bootstrap nMDS means and 95% confidence ellipses of bacterial and archaeal communities based onBray-Curtis similarities. A) Extraction blanks, coupon type and water samples B) Extraction blanks, sampling dates and water samples C) Extraction blanks, sample site and water samples.

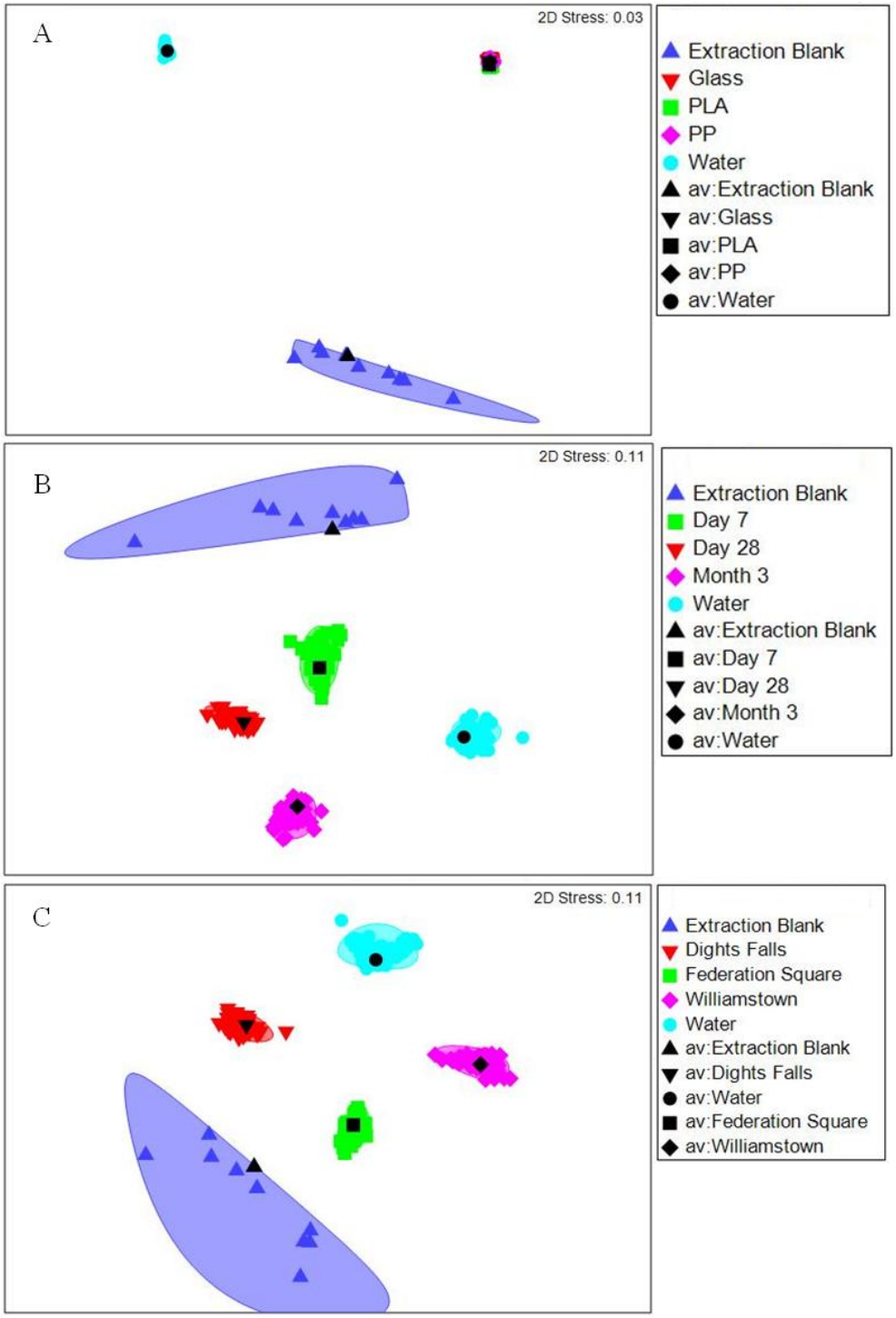


Figure 5.17 Non-metric multidimensional scaling (nMDS) representation of 60 bootstrap nMDS means and 95% confidence ellipses of eukaryotic communities based on Bray-Curtis similarities. A) Extraction blanks, coupon type and water samples B) Extraction blanks, sampling dates and water samples C) Extraction blanks, sample sites and water samples.

5.4 Discussion

This study investigated variation in microbial community diversity, structure and taxonomic composition along a freshwater-marine continuum of the Yarra River, Melbourne, over a 3 month period for PP-, PLA- and glass- microbial biofilm communities and free-living/particulate associated water communities.

The diversity and evenness of the microbial communities was assessed by calculating the number of OTUs, Shannon and Simpson diversity and Pielou evenness metrics. The mean number of observed OTUs, diversity and evenness were consistently lower for both the prokaryotic and eukaryotic biofilm communities when compared to the water communities (except for the prokaryotic communities at the Williamstown site) (Table 5.4 and Table 5.5). These findings were consistent to those from bacterial communities that were sourced directly from plastic samples in freshwater environments (McCormick *et al.*, 2014, McCormick *et al.*, 2016), and from fungal communities on PS and PE that had been deployed in seawater for 15 days (Kettner *et al.*, 2017). However, the diversity and evenness of the microbial communities in the Yarra River contrasted with the bacterial communities that were present in marine sediments in the North Sea (De Tender *et al.*, 2015); in which the diversity and species richness of plastic biofilm communities were greater than in the free-living water communities but lower than in the sediment biofilm communities. Whilst the diversity of the biofilm communities(using the mean from all five sites of this current study) was lower for both the prokaryotic and eukaryotic biofilm communities when compared to the diversity of water communities; diversity was higher in the marine (Williamstown) biofilm communities when compared to the water communities. This is consistent with the findings by Dussud *et al.* (2018), for prokaryotic biofilm communities on PP and PS samples recovered directly from the Mediterranean Sea.This suggests that more bacterial taxa may be able to form biofilms in marine environments when compared to freshwater environments. Eukaryotic

communities had lower OTUs, diversity and evenness in the biofilm communities when compared to the water communities at the Williamstown site, which was consistent with studies of plastic biofilm fungal community diversity and evenness in seawater when compared to free living communities in water itself (Kettner *et al.*, 2017). Pearson Correlation analysis to compare diversity and Chao1 OTU richnessin the aggregatedeukaryotic and prokaryotic biofilm and water microbial communities to the water physico-chemical electrical conductivity and pH data (see Section 5.3.3.1), identified only one significant relationship ($P < 0.001$) namely, a positive relationship between the numbers of observed OTUs for the 16S rRNA gene sequences from the biofilm communities with pH. This contrasts with Herlemann *et al.* (2011) who found there was a positive correlation between electrical conductivity and diversity of backterioplankton along a 2,000 km long salinity gradient. However, the Herlemann *et al.* (2011)study was conducted in marine water in the Baltic Sea and did not use freshwater samples, in contrast to this current study.

Variation in the structure of microbial communities was compared using neighbor-joining trees constructed using Bray-Curtis dissimilarity matrixes. Both prokaryotic and eukaryotic community structure primarily correlated with the sample being either from water or biofilm communities; secondly by sample site and thirdly by sampling date (see Section5.3.3.2). These findings of microbial community structure being highly differentiated based on sample location were consistent with previous studies comparing floating plastic bacterial biofilm communities from different marine locations (Zettler *et al.*, 2013, Bryant *et al.*, 2016, Oberbeckmann *et al.*, 2016, Oberbeckmann *et al.*, 2018), marine sediments (De Tender *et al.*, 2015), and freshwater environments (Hoellein *et al.*, 2014, McCormick *et al.*, 2014, McCormick *et al.*, 2016) and for marine plastic fungal biofilm communities (Kettner *et al.*, 2017) and algal communities (Oberbeckmann *et al.*, 2014, Oberbeckmann *et al.*, 2016). Of these earlier studies, only four used field-based exposure experiments (Hoellein *et al.*, 2014,

Oberbeckmann *et al.*, 2016, Kettner *et al.*, 2017, Oberbeckmann *et al.*, 2018), and none of these reported data from different stages of the biofilm formation process. To date, there has been only one study which has assessed the microbial biofilm formation process on plastic in a marine environment (Toulon Bay, France) (Pollet *et al.*, 2018), which was conducted over a 75 day period. The authors reported no consistent patterns in either the Shannon diversity or Chao1 richness after Day 4, with the lowest diversity and Chao1 estimate being from the Day 1 samples.

To investigate whether the structures of microbial biofilm communities would converge over time, SIMPER analysis was conducted. No significant difference ($P > 0.05$) was found between the structures of the prokaryotic communities between sampling dates. For the eukaryotic communities, a significant difference ($P < 0.05$) between community structures at different time points was found for the Federation Square samples only. However, this difference was caused by the communities becoming less similar to each other which was contrary to the expectation that the communities would become more similar. Two crustacean families, Harpacticidae and Misophriidae, were detected only on sample S3G3M3 of the biofilm samples from Federation Square (see Section 5.3.7.2.2). These are multicellular organisms and relatively large when compared to the other unicellular eukaryotic organisms associated with the plastic biofilms such as diatoms and ciliates. Therefore, a potential bias arises towards the relative contributions of multicellular organisms when compared to single cell organisms when estimating abundances of multicellular organisms (where each cell contains at least one rRNA gene), upon which the Bray-Curtis matrixes are based (Medinger *et al.*, 2010, del Campo *et al.*, 2014). Non-metric multidimensional scaling (nMDS) of the community structural similarities using both the Bray-Curtis and Jaccard dissimilarity (based on a presence/absence transformation) matrixes (Figure 5.8), indicated that biases arising from taxa being multicellular or having multiple 16S or 18S rRNA genes per cell did not

impact upon the interpretation of the results. The factors that are different between the study by Harrison *et al.* (2014), who initially hypothesised the potential for biofilm community convergence, and this current study are; the previous study deployed plastics into sediments, used microcosms for up to 14 days while in this current study plastics were deployed on the water surface, and used a direct field exposure study *in situ* and had a much longer duration of up to 3 months.

Variation in microbial community structure between the three different substrate types (PP, PLA and glass) was assessed. No significant difference ($P > 0.05$) was found between the eukaryotic biofilm communities present on the three substrate types at any of the three sites and three sampling dates. A significant difference ($P < 0.05$) was found for prokaryotic communities, but only in one instance(Site 4, Day 14) out of 51 tested combinations, between glass and polypropylene,(S4GD14and S4PPD14). This result is likely to be stochastic and a type II error. Therefore, the null hypothesis should be accepted that the plastic-associated biofilm communities were the same as the glass-associated biofilm communities. These findings demonstrate that microbial communities on bioplastics (PLA) and traditional petroleum-based plastics (PP) are likely to be the same, under the same environmental conditions. This is consistent with previous studies comparing plastic biofilm communities to other hard-substrates such as glass and ceramic tiles in freshwater (Hoellein *et al.*, 2014), and in comparison to glass in marine environments (Oberbeckmann *et al.*, 2016) which found that the plastic biofilm communities were very similar to the other hard-substrate biofilm communities. Plastic biofilm communities have been found to be different when compared to soft-surface substrates such as leaves and wood (Hoellein *et al.*, 2014, Kettner *et al.*, 2017, Dussud *et al.*, 2018, Oberbeckmann *et al.*, 2018). This may be due to microorganisms that form biofilms on plastics in aquatic environments benefitting from the same processes that

other biofilms in aquatic environments have, such as cell-cell interactions and proximity to resources which adhere to particles through electrostatic forces (Dang *et al.*, 2018).

To date, the archaeal component of prokaryotic biofilm communities have not been reported. This may be because they form part of the 'rare biosphere', contributing <0.5% of the DNA sequence reads, and are therefore ignored when considering the more abundant organisms present in a community. The bacterial phyla that dominated the prokaryotic communities were Proteobacteria and Bacteroidetes. Proteobacteria and Bacteroidetes have frequently been found to be dominant bacterial phyla in plastic biofilms from freshwater environments (Hoellein *et al.*, 2014), marine water (Oberbeckmann *et al.*, 2014, Bryant *et al.*, 2016, Dussud *et al.*, 2018, Oberbeckmann *et al.*, 2018, Rampadarath *et al.*, 2017) and marine sediment environments (De Tender *et al.*, 2015). Cyanobacteria were not detected above a relative abundance of 1 % in any of the biofilm communities of this study but have been frequently detected in previous plastic biofilm studies, notably as dominant taxa from samples sourced from open-water marine environments, all of these studies used samples that were directly collected from either the Mediterranean Sea (Bryant *et al.*, 2016, Dussud *et al.*, 2018)or the North Sea(Oberbeckmann *et al.*, 2014). In marine coastal waters, Cyanobacteria tend to be outcompeted by photosynthetic eukaryotes such as algae, diatoms and dinoflagellates (Paerl Hans, 1988, Smith, 2003).

The family Rhodobacteraceae (Alphaproteobacteria) had the highest relative abundance within the biofilm prokaryotic communities. Members of the Rhodobacteraceae have regularly been observed as the most dominant bacterial family in marine biofilms, such as on glass surfaces (Elifantz *et al.*, 2013) and as symbionts associated with microalgae (Wagner-Döbler *et al.*, 2009, Amin *et al.*, 2012). Rhodobacteracae consist of both phototrophic and heterotrophic taxa (Shalem Raj *et al.*, 2013). A constituent genus of Rhodobacteraceae is the *Roseobacter spp.*, which are found to be common and widespread as marine surface

colonisers including on corals, faecal pellets and shellfish (Luo and Moran, 2014, Dang and Lovell, 2016). In the biofilm communities in this current study, amongst the Rhodobacteraceae family, *Oceanibulbus spp.*, *Primorskyibacter spp.* and *Celeribacter spp.* had the highest relative abundances, and are closely related to *Roseobacter spp.*(Wagner-Döbler *et al.*, 2004, Ivanova *et al.*, 2010, Romanenko *et al.*, 2011). Pseudomonadaceae have previously been found to be dominant taxa in freshwater plastic debris biofilms (McCormick *et al.*, 2014, McCormick *et al.*, 2016); however, they were not found to be present at relative abundances of >1% in any of the biofilm samples from this current study.

The eukaryotic biofilm communities were dominated by Bacillariophyta (diatoms) (see Section5.3.7.2.2), which are known to be prevalent surface colonisers of natural debris such as macro-organisms and rocks (Salta *et al.*, 2013), as well as plastic (Reisser *et al.*, 2014, Oberbeckmann *et al.*, 2016). The second most common eukaryotic taxa were Vorticellidae (ciliates), but only in the Dights Falls and Williamstown communities. To date, Vorticellidae have not been reported as a dominant eukaryotic family on plastic debris. In the Dights Falls biofilm communities, the Vorticellidae taxon with the highest relative abundance was *Vorticella sp.* (Accession number: JX178764), which was first described by Gong *et al.* (2013). They determined that *Vorticella* (Accession number: JX178764) had 82,194 copies of rDNA (rRNA genes) per cell. Therefore it is unlikely that *Vorticella sp.* were as prevalent as the 18S rRNA gene data suggests. As discussed previously, there was no effect from the bias of multicellular organisms or organisms with multiple rRNA genes on the analysis of community structure in this particular study as determined by comparison using Bray-Curtis and Jaccard methods (Figure 5.8). However, caution is needed when trying to determine the most dominant taxa within a community from rRNA gene data alone. Issues and biases relating to amplicon sequencing have been extensively explored in a number of research articles and reviews. Biases arise with the PCR process itself due to preferential amplification

(Suzuki and Giovannoni, 1996, Wintzingerode *et al.*, 1997). Moreover, biases can arise from contamination during the DNA extraction process, especially when extracting from a low biomass source material (Salter *et al.*, 2014, Glassing *et al.*, 2016). Additional biases are due to taxa containing varying numbers (copies) of rRNA genes (Schloss *et al.*, 2011). Whilst biases and issues associated with regards to the choice and use of bioinformatics pipelines and taxonomic databases such as uneven distribution of taxa identified in the databases can also further impact on interpretation (Plummer *et al.*, 2015, Balvočiūtė and Huson, 2017).

This was the first study to assess the development of biofilm communities on plastic debris via a field base exposure experiment over at least a 3 month period along a freshwater-marine continuum. Both the prokaryotic and eukaryotic communities changed over the three month period of the field trial. Flavobacteriaceae, Sphingomonadaceae and Erythrobacteraceae increased in relative abundance between Day 7 and Month 3 and all three bacterial families have been found to be with epiphytic on Chlorophyta (Green algae) (Glaeser and Kämpfer, 2014, Florez *et al.*, 2017), which also increased in relative abundance at Federation Square between Day 7 and Month 3. Flavobacteriaceae have also been found to increase in relative abundance in marine plastic biofilm communities after 4 days of being exposed to seawater (Pollet *et al.*, 2018). No common explanatory factor to account for the changes in eukaryotic composition was able to be deduced.

To date, most studies that have identified bacterial and fungalspecies that can potentially degrade plastics have used laboratory methods and have used soils as the source material (see Section 1.5.2). There has been no prior attempt to screen plastic biofilm communities from aquatic environments against these known potential plastic degraders. Of the 75microorganism species identified (43 bacteria and 32 fungi) with potential plastic degrading capabilities, only the bacterium *Bordetella petrii* and the fungus*Aspergillus fumigatus* wereidentified in this current study. Both *B. petrii* and *A. fumigatus* were

frequently detected (94 % and 58 %, respectively). However, both taxa were also part of the rare biosphere, having relative abundances of<0.1 %. Therefore, it is unlikely that either *B. petrii* or *A. fumigatus* contributed to any substantive biodegradation of the plastic coupons.

It has previously been suggested that plastics are potential reservoirs of pathogenic bacteria and harmful algae (Zettler *et al.*, 2013). In this current study, of the 758 pathogens screened for, only 31 pathogens were identified within the biofilm communities (*Appendix V*). The most frequently identified pathogens were *Pseudomonas anguilliseptica*, *Acinetobacter johnsonii* and *A. lwoffii*, all of which are known fish pathogens (Berthe *et al.*, 1995, Kozińska *et al.*, 2014). However, only *P. anguilliseptica* had a substantially higher maximum relative abundance in the biofilm communities when compared to the water communities (11.6%). *P. anguilliseptica* is known to infect wild fish (Doménech *et al.*, 1997), and is associated with high mortality rates in eels (*Anguilla juponica*), black sea-bream (*Acanthopagru.s schlegeli*) and sea-bass (*Dicentrarchus labrax*) (Nakajima *et al.*, 1983). *A. johnsonii* and *A. lwoffii* have only been reported to infect farmed fish (Kozińska *et al.*, 2014), and have been found to be resistant to a range of antibiotics including oxytetracycline, ampicylin and amoxycylin (Kozińska *et al.*, 2014, Miranda and Zemelman, 2002). These data indicate that marine plastic debris may offer a pathway for exposure off fish to pathogens.

Both the plastic and water biofilm communities had very low relative abundances of harmful algae (<0.07%). These data suggests that aquatic plastic debris may not likely have a high likelihood of causing the spread of, or act as reservoirs of, harmful algae. However, this study occurred between June and September, representing the austral winter and early spring. Algal blooms are known to develop in warmer waters during late spring and summer (Alldredge and Gotschalk, 1989, Fry and Wainright, 1991). Therefore, investigation of algal colonisation in summer months is warranted, via collection of samples during the peak algal blooming period.

To conclude, in this chapter it was shown that microbial biofilm communities on plastic are similar in taxonomic composition and structure to those on other hard surfaces such as glass. These biofilm communities undergo distinct community dynamics, and community structure and taxonomic composition is predominately influenced by the source location, i.e. freshwater vs. marine water locations. Known potential plastic-degrading microorganisms and hazardous algae taxa had very low relative abundances (<0.1%) among the biofilm communities. In addition, three fish pathogens were identified in >75% of biofilm samples, with relative abundances up to 11.6%, indicating that plastics may be a potentially novel pathway for pathogen exposure to marine life.

Chapter 6: General Discussion

The aim of the research in this thesis was to compare the fates of two consumer-based plastic item types; take-away containers made from traditional petroleum-based plastic [polypropylene (PP)] and coffee cup lids made from a model bioplastic [polylactic acid (PLA)]within and across multiple aquatic environments during a 12 month exposure experiment. There were three key aspects in this research, namely: 1) to investigate the variation in the structural properties of PLA and PP via analysis of surface hydrophobicity, tensile strength, crystallinity and chemical structure 2) determine the potential for brominated flame retardants (polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs)) to accumulate onto PP and PLA with comparison to glass substrates 3) compare spatial-, temporal- and substrate-specific (PP, PLA and glass) variation in the structure and composition of microbial (prokaryotic and eukaryotic) substrate biofilm communities and also in relation to those communities in surrounding water. The key findings were firstly that PLA coupons were as physically and chemically stable over a 12-month period in aquatic environments as the petroleum-based PP coupons. Secondly, at least one PBDE and NBFR were present on every sample analysed;Penta-BDEs (BDE-99 and BDE-100) and the NBFR compound, pentabromotoluene (PBT), were detected in 95 % and 84 % of exposed coupons (PLA, PP and glass), respectively and suggested that ingested PLA could pose a similar level of risk to marine organisms for transporting and enabling exposure to hydrophobic organic chemicals (HOCs) as petroleum-based plastics such as PP, polyethylene (PE) and polystyrene (PS) in natural aquatic environments. Thirdly, the microbial biofilm communities were distinct from those in the surrounding water, but were highly similar in taxonomic composition and structure between all three substrate types (PP, PLA and glass); with community structure and composition principally discriminated firstly by sample location and secondly by sampling date. Finally, the relative abundance of

functional guilds of potential plastic degraders, harmful algae and pathogens werelargely negligible (<1 % of 16S and 18S rRNA gene amplicon reads) within these communities.

Research in Chapter 3 compared variation in the physical and chemical properties of the PLA and PP coupons through multiple approaches, namely: surface hydrophobicity, tensile strength, crystallinity and chemical structure. Neither coupon type changed substantially over the 12-monthexposure period in regards to these properties. This is an important finding as it demonstrated that PLA was as physically and chemically stable as PP for up to a yearin aquatic environments.The current study was conducted over an extensive period of time (12 months) compared to previous comparable *in situ* studies of 5 weeks (Tsuji and Suzuyoshi, 2002) and 32 weeks (Weinstein *et al.*, 2016).Due to an uneven distribution of replicates, due to loss of samples from sampling frames, samples from each locationfor each sampling date were combined together for statistical analysis; this approach may have concealed site-specific differences that arose between the different sampling locations, as there is some evidence that degradation of polymers is effected by salinity (Da Costa *et al.*, 2018). However, since the data generated (WCA, tensile strength, crystallinityand FTIR spectra)were broadly consistent for each sampling time point, any effect due to site-specific variation is likely to be small. These results are important for policy makers; as it has been demonstrated that bioplastics are just as recalcitrant in aquatic environments as traditional petroleum-based plastics over a 12-month period.PLA should therefore be treated with the same regard as traditional petroleum-based plastics such as PP, PE or PS when developing and implementing policies in relation to plastic production, use, pollution and waste management (United Nations Environment Programme, 2018a).

Research in Chapter 4 sought to detect and quantify PBDEs and NBFRs thathad accumulated over a 12-month period onto PLA, PP and glass substrates that were placed along a freshwater-marine continuum. Samples were lost from coupon sampling frames over the

course of the exposure period and therefore limited the capacity for comparison between hydrocarbon (PBDE and NBFR) accumulation onto the coupons.The analysed PBDEs and NBFRs were present on all samples. Concentrations of PBDEs and NBFRs detected on the polymer and glasscoupons were found to be extremely small, with most detected congeners being present at less than the method quantitation limit (MQL) concentrations. By Month 12, only in 18 out of a possible 56 instances were the concentrations of PBDEs above the MQLs (4 out of 28 instances for the selected NBFRs). Nevertheless, several samples had substantial concentrations of ∑PBDEs (M12S1L3: 270 ng g^{-1}, M12S1L4: 70 ng g^{-1}, M12S1L5: 55 ng g^{-1}). PBDEs have previously been quantifiedon plastic recovered from the Central Pacific Gyre within a range of 0.02 ng g^{-1} - 9,900 ng g^{-1}(Hirai *et al.*, 2011) and 0.6 ng g^{-1} -188 ng g^{-1}(Chen *et al.*, 2018), respectively. Those plastics were likely present in the marine environment for longer than 12 months, and therefore had a longer period for PBDEs to adsorb onto their surfaces. Due to their hydrophobic properties, PBDEs and NBFRs are more likely to be bound to sediments than to be present in the aqueous phase in aquatic environments (Yue and Li, 2013). The presence of thick biofilms may also inhibit the direct adsorption of POPs onto the plastic hydrophobic surface, since biofilms have been demonstrated to alter the adsorption behaviour of metals onto plastic surfaces, or alternatively and conversely allow accumulation into the biofilm extracellular polymeric substances (EPS) directly or via accumulation of sediments with adsorbed POPs within the biofilm matrix(Ashton *et al.*, 2010). The relative proportions of POPs that are partitioned between plastic biofilms or that are directly associated with the plastic surface remains to be investigated.

Research in Chapter 5 explored comparison of the microbial biofilm communities on PLA, PP and glass substrates and the microbial communities from water samples. The microbial communities were distinct in terms of community taxonomic structure and compositionfirstly between biofilm and the free–living water communities, secondly between the sample

locations and thirdly between sampling dates. However, the communities were highly similar to each other when considering differences between different coupon types sampled from the same date and location. Within these communities, only two known potential plastic-degraders were detected, the bacterium *Bordetella petrii* and the fungus *Aspergillus fumigatus*.However, both *B. petrii* and *A. fumigatus*were part of the rare biosphere, having relative abundances of <0.1 %. In addition, three fish pathogens, *Pseudomonas anguilliseptica, Acinetobacter johnsonii* and *A. lwoffii*, were frequently identified, being detected in over 100 of the biofilm communitieson polymers (and glass) suggesting a possible route for pathogen transport in the aquatic food chain. To the best of my knowledge this is the longest study into biofilm formation onto plastic in natural aquatic environments (3 month duration) using high-throughput DNA sequencing techniques; with the most samples across different aquatic environments including freshwater, estuarine and marine waters, and the first to simultaneously compare microbial biofilms from a petroleum-based plastic, a bioplastic and a non-plastic hard surface substrate. This allowed for greater insights into the taxonomic composition and structure of mature, complex biofilm communities than previously achieved.

The microbial biofilm communities had highly similar compositions between the plastic (PP and PLA) and the non-plastic substrates(glass). This draws into question the appropriateness of the term "*Plastisphere*" which was first termed by Zettler *et al.* (2013) claiming that a 'core' group of plastic-specific taxa colonised plastic debris. In that early study, thesample size was comparatively small, with only six samples and no non-plastichard substrates (control)wereanalysed(Zettler *et al.*, 2013).However, there was no significant distinction between the diversity, structure or composition of microbial biofilms on plastics when compared to communities on other hard surfaces such as glass in this current studyfrom freshwater and marine environemnts or in a previous study in the marine enivornment

(Oberbeckmann *et al.*, 2016)oron ceramic tiles from freshwater environments(Hoellein *et al.*, 2014).It is likely that marine plastics offer biofouling microorganisms the same advantages as other substrates when compared to existing in the free-living picoplankton, principally via increased access to nutrients which tend to adhere to surfaces via such means as electrostatic forces (Dang and Lovell, 2016). However, since plastic is, by far, the most frequently retrieved anthropogenic debris type in marine litter surveys and the amount of plastic now floating in the oceans is so large (van Sebille *et al.*, 2015), that the biofilm communities are distinct from the free-living communities (Oberbeckmann *et al.*, 2018), the term "*Plastisphere*" is still appropriate when refering to plastic biofilm communities.

Most of the taxa identified in the literature as being associated with plastic biodegradation have been isolated from soils, and therefore may not be suited to live on plastics in aquatic freshwater or marine environments (see Section1.5.2). None of the marine organisms that have previously been found to degrade plastics were identified in this current study; these microorganisms included the bacterium *Ideonella sakaiensis* that was isolated from marine sediments (Yoshida *et al.*, 2016), which was found previously to be able to degrade low-crystalline PET and the bacterium *Pseudomonas alcaligenes*(Kim *et al.*, 2005) that had been isolated from seawater, or the marine fungus *Zalerion maritimum*(Paço *et al.*, 2017). Similarly, De Tender *et al.* (2015) also did not identify any of the known marine plastic degraders on plastics recovered from marine sediments from the North Sea. This suggests that the abundance and frequency of plastic degraders on plastics in aquatic environments is very low.

In comparison to microbial communities which develop in response to marine crude oil-spills(Almeda *et al.*, 2014), plastic biofilm communities are complex, consisting of diverse taxa including photosynthetic and heterotrophic bacteria, diatoms, algae and protozoa (Bryant *et al.*, 2016). Crude oil is highly toxic to many microorganisms and crude oil spills can

severely impact indigenous microbial communities (Kostka *et al.*, 2011). Therefore, as a result of an oil spill, microorganisms that were part of the rare biosphere and are adapted to crude oil intrusionsmay increase in their relative abundance(Kleindienst *et al.*, 2015), with consequently less competition for nutrients such as nitrate and phosphate (Liu *et al.*, 2016). However, there are challenges for these microorganisms to access these nutrients that tend to remain in the aqueous phase and do not enter the organic phase readily (Prince and Atlas, 2018). These challenges can be overcome by the microorganisms producing biosurfactants which increase the bioavailability of oil products, and thereby increase microbial growth based on oil (Ron and Rosenberg, 2002, Shekhar *et al.*, 2015). In comparison, plastics are relatively bioinert, resulting in no large adverse impacts to microbial communities due to the presence of plastic in water.In biofilm communities, extracellular polymeric substances (EPS) are produced by bacteria, algae and diatoms (Xiao and Zheng, 2016). The EPS are complex structures that contain polysaccharides, proteins and lipids which can potentially provide a more readily available energy source for the embedded microorganisms when compared to the more recalcitrant polymer molecules in plastics (Zhang and Bishop, 2003, Flemming and Wingender, 2010). Therefore, organisms that have been associated with polycyclic aromatic hydrocarbon (PAH) and crude oil degradation such as the bacterium *Pseudomonas putida*(Bastiaens et al., 2000) and the fungus *Aspergillus oryzae*(El-Hanafy et al., 2017), respectively, as well as plastic degradation are potentially being out-competed by microorganisms that are able to utilise the more bioavailable and energetically more favourable organic carbon compounds present within the EPS.

Plastics debris may travel long distances and through many different environments once it has been littered, travelling through gutters and storm drains, to urban rivers, being trapped in sediments and in the riparian zone to then ultimately flow out to sea. Residence in these distinct habitats may have long lasting effects on the plastic. For example, plastics may be

exposed to intense sun-light while caught on a high thermal mass material such as a pavement,beach or shoreline, thereby leading to an increased rate of thermo-photo-oxidative degradation. Once an initial (partial) degradation process of the plastic and plastic additives been initiated, the plastic object may become embrittled and therefore more likely to disintegrate, creating microplastics(Andrady, 2011). Also, through this journey, the plastic may come into contact with substances or surfaces that contain a high concentration of POPs such as road side dust and may transfer onto the plastic surfaces. For instance, PBDEs have been quantified in roadside dust within a range of 0.91 ng g^{-1}-56 ng g^{-1}(Anh *et al.*, 2018). There are also implications for the microbial community development as well, particularly for those from the estuarine MacRobertson Bridge and Federation locations and the marine Westgate Bridge and Williamstown locations, since many freshwater microorganisms are unable to persist in environments of elevated salinity (Painchaud *et al.*, 1995). This is because microbial communities would have already developed on the plastics while the plastics were still moving through stormwater drains and the freshwater reaches of the river before entering the estuarine reaches. Therefore, the succession in the plastic microbial biofilm communities as they change from soil and freshwater based communities to estuarine and marine based communities with different functional characteristics needs to be further studied.

As noted by De Tender (2017), only one biofilm development cycle was undertaken, hence, the co-variables of biofilm development stage and season could not be differentiated. To date, only two studies investigating seasonal variation in plastic biofilms have been conducted(Oberbeckmann *et al.*, 2014, Oberbeckmann *et al.*, 2016); the authors reported seasonal variation in both the prokaryotic and eukaryotic microbial communities. Therefore, this current study could be extended by starting the deployment of the coupons in multiple and different seasons. This would allow for insights with regards to the presence and relative abundance of harmful algae, as algal blooms occur most often in summer (Longhurst, 1995),

while this microbial biofilm formation study was conducted over the austral winter. Potentially the single most practical way the experimental design of the studies of this thesis could have been improved, would have been to use thicker fishing line that the samples were threaded to. Sample loss, particularly for the latter months of Month 6, Month 9 and Month 12 due to the fishing lines breaking restricted analysis of later time points across sites and substrates (Chapter 3 and Chapter 4).

PLA has a density of 1.25 g cm^{-3}, while freshwater has an approximate density of 1.0 g cm^{-3} and seawater an approximate density of 1.3 g cm^{-3}. Therefore future research into PLA degradation, POP accumulation or biofilm formation should compare water surface samples with samples embedded within the sediments. In addition, the way that the sample frames were designed, purposefully prevented the plastic substrates from sinking. Therefore, even when the substrates were covered in thick eukaryotic biofilms containing molluscs, bivalves and bryozoa, the plastic were still suspended on the water surface. This hindered the natural process of the plastic sinking to the sediments, where potentially different organisms could have been able to colonise the plastics. The substrates used were also quite large in size, 54.7 cm^2 (PLA), 73.0 cm^2 (PP) and 27.5 cm^2 (glass), compared to the microplastics used in previous studies that are often millimetres in size (Reisser *et al.*, 2014, Dussud *et al.*, 2018, Oberbeckmann *et al.*, 2018). Larger plastic fragments are able to support larger eukaryotic organisms such as mussels (bivalvia) and sea squirts (ascidians) when compared to microplastics (Figure 6.1). These larger eukaryotic organisms may have specific epibiont microbial communities associated with them (Wahl, 1995, Taylor *et al.*, 2003), and thereby be specific to the plastic biofilm community as a whole. For example Taylor *et al.* (2003)found that microbial biofilm communities associated with three species of sponges(*Cymbastela concentrica*,*Callyspongia sp.*and *Stylinos sp.*) varied little withineach species of sponge, but microbial community varied substantiallybetweenthe sponge

species.Future research into the microbial composition of aquatic plastic debris should compare microplastics with larger macroplastic substrates.

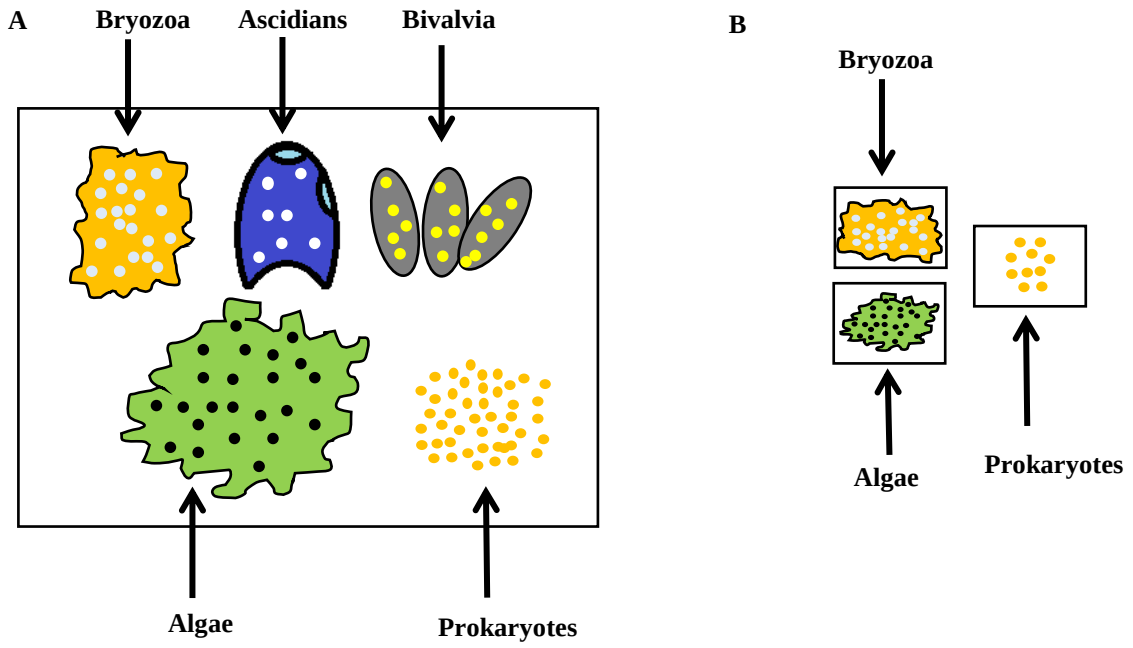


Figure 6.1Conceptual diagram of differences inbiofilm community taxonomic composition on plastic substrates (rectangles) with their associated distinct epibiont communities (circles) between A) Macroplastics and B) Microplastics. The microplastics are not large enough to support colonisation by larger eukaryotes and their associated epibiont communities, such as ascidians and bivalvia.

Plastics in this current study were readily colonised by photosynthetic organisms such as diatoms and green algae (See Section 5.3.7.2.2). In a previous studyof plastic in the North Pacific Subtropical Gyre, plastic biofilms of a similar composition to this current study also had chlorophyll-*α*measured as being equivalent to those present with volumes of 30 mL to 700 mL in the surrounding seawater(Bryant *et al.*, 2016). Those waters were largely oligotrophic and so have generally low chlorophyll-α concentrations (Van Mooy *et al.*, 2006). Similarly, from plastic bags suspended 25 m below the water surface in the Mediterranean Sea the mean net oxygen production of biofilm communities (measured*in vitro*) from the

plastic bags was 0.17 μmol^{-1} $h^{-1}cm^{-2}$(Pauli *et al.*, 2017). Although Pauli *et al.* (2017) concluded that the production of oxygen in the Mediterranean Sea from plastic biofilms communities was inconsequential when compared to that from the free-living water communities due to themuch higher surface area of water when compared to the surface area of all plastic debris, the net productivity of $O_2$from plastic biofilmcommunities was greater than that of gross O_2production from comparable water oligotrophic regions of the north-west Mediterranean Sea (0.028 μmol^{-1} $h^{-1}L^{-1}$- 0.20 μmol^{-1} $h^{-1}L^{-1}$) in a different study(González *et al.*, 2008). The O_2 production results from Pauli *et al.* (2017)study were from plastics kept 25 m below the water surface and therefore may not represent microbial communities present on plastics at the water surface.In addition, as biofilms develop on plastics debris, macroplastic debris (Pauli *et al.*, 2017) and microplastic debris (Kaiser *et al.*, 2017) may sink, whereby the biofilm community is transported to the seafloor (Figure 6.2). Figure 6.2 is a reduced net transfer model and does not depict interactions such as grazing of biofilms or cell lysis due to viruses. Evidence is gathering in support of the hypothesis that the seafloor is the ultimate destination for marine plastic debris (Ling *et al.*, 2017, Chiba *et al.*, 2018). Therefore, estimating the global productivity of plastic pollution as well as the biomass on plastic debris as it sinks in marine environments may be worthwhiledue to the amount of plastic present in the ocean,approximately 15- 51 trillion particles; van Sebille *et al.* (2015).Approximately 3.41 trillion of these plastic particles are greater than 1 mm in size; Eriksen *et al.* (2014), as marine plastic debris may be a new, important vertical sink for CO_2in the oceans in addition to sinks fromcarbonate and particulate matter transport (Volk and Hoffert, 1985, Honjo *et al.*, 2008).

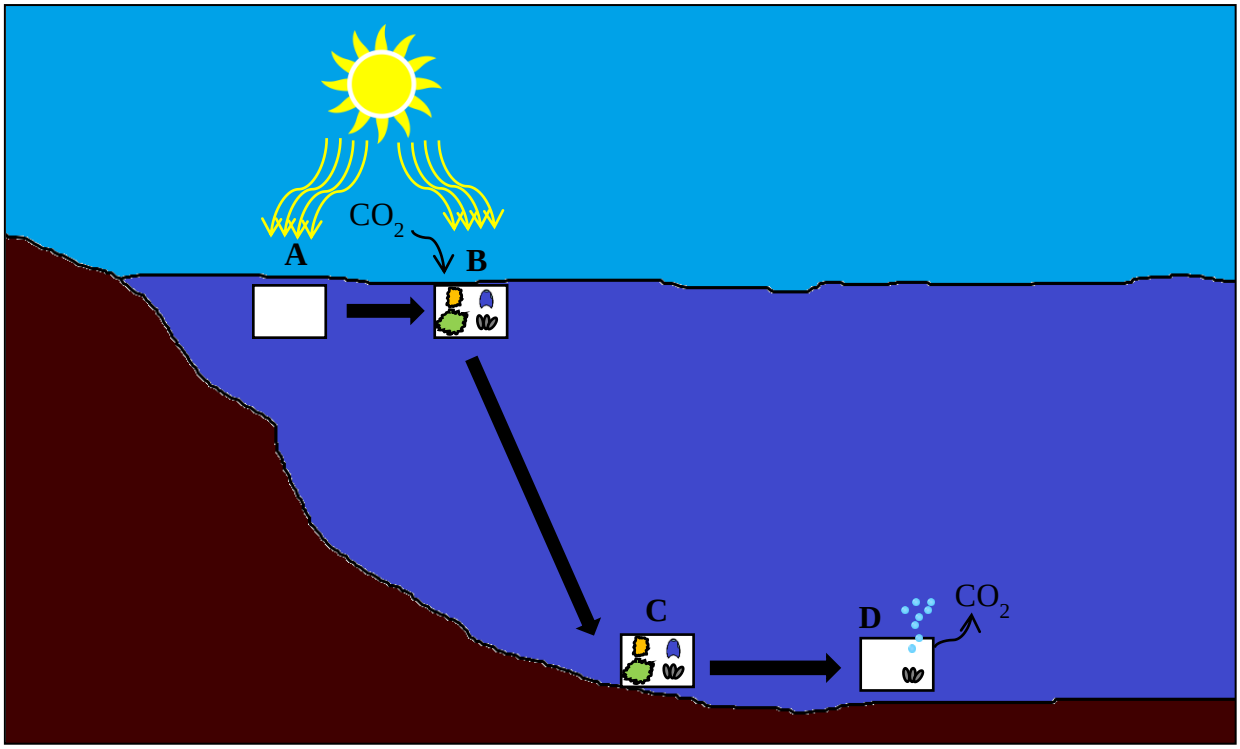


Figure 6.2 Conceptual diagram of the net transfer ofbiofilm biomass on plastic (rectangles) from surface waters into benthic environments whereby: A) Biofilms develop on plastic surfaces in aquatic environments B) The mass of the biofilm continues to grow until the plastic sinks to the seafloor C) The organisms in the biofilm perish being unadapted for the new conditions D) Microbial respiration from the degradation of the plastic biofilm releases CO_2into the surrounding water.

Currently there is a substantial disparity between the actual life of single-use consumer plastic items and the intended time in which they are designed to be used. For instance, the physical and chemical properties of PLA coffee cup lids used in this thesis were found to be highly stable in aquatic environments for at least one year, yet coffee cup lids are used by consumers in the order of minutes. Rani *et al.* (2015) identified 231 different chemicals on plastic surfaces recovered from beaches, a broad range of these chemicals were plastic additives such as UV stabilisers, antioxidants and phthalates. Once plastic additives are leached from the

polymers, the polymers may become embrittled and disintegrate (Andrady, 2011). Identifying and developing plastic additives which are eitherbiodegradable or degradable by other means such as POD in marine environments, may lead to industry preferentially using these additives over alternatives. To be sucessful, these additives would need to be economical to produce, non-toxic, not migrate from the polymer and not leadto degradation of the polymer during manufacturing or processing (Cassidy and Aminabhavi, 1981) and avoid compromising the physical and chemical propertises of the item as can occur with co-polymer composities such as high starch content plastic blends (Luckachan and Pillai, 2011). This may increase the disintegration rate of plastics in aquatic environments and increase the rate in which the plastic particles settle out of the water column and into the marine sediments, reducing the risks of transport to larger pelagic marine organisms. However, the impact of small plastic fibres and particles in marine water and benthic communities is still poorly understood. Although a previous study investigating degradation rates of plastics embedded with biodegradation-promoting additives did not find evidence to suggest that these chemicals increased polymer degradation (Selke *et al.*, 2015); only five different chemicals were tested, and information about the modes of action were not provided. Therefore there is still a need for further research into degradation-promoting additives in plastics that may reduce the structural life-span of short-term used in single use plastic items.

Finally, a study was recently published that demonstrated the fish fry had a reduced predatory flight response when microplastics were present in environmentally relevant concentrations in water (Lönnstedt and Eklöv, 2016). The authors suggested this reduced response occurred because the plastics altered the olfactory cues of the fish. However, this study was retracted on May 26, 2017, because of controversies involving scientific misconduct. Another controversial paper (Savoca *et al.*, 2016, Dell'Ariccia *et al.*, 2017) investigated the olfactory response in seabirds to the dimethylsulfide (DMS) produced by algae in the plastic biofilms.

The authors suggested that an olfactory response may be why some seabirds appear to selectively forage for plastics. With these studies are disputed, there is a need for more studies into the potential behavioural impacts of plastic pollution on marine organisms. Key research questions should re-examine the above studies, as well as investigating other possible behavioural effects of plastics and/or plastic additives to aquatic organisms.

This thesis aimed to advance the current knowledge of the fate of pollutant plastics within aquatic ecosystems by investigating changes in the physical and chemical structure of a petroleum-based plastic and a bioplastic. PLA has been shown to be as stable as PP over a 12-month period in aquatic environments, and has the capability to accumulate PBDEs and NBFRs. The plastic biofilm communities were shown to be diverse and distinct from the surrounding water communities, and that the plastic biofilm communities were highly similar to those forming on glass, demonstrating that plastic biofilm communities consists of predominantly generalist surface colonisers. Although the relative abundance of pathogens in the biofilm communities were low, three fish pathogens (*Pseudomonas anguilliseptica,Acinetobacter johnsonii* and *A. lwoffii*) were present in over 100 coupon samples indicating that aquatic plastic debris may be a novel exposure pathway of pathogen exposure in fish due to the high number of plastic fragments in aquatic environments, and the ability of plastics to passively travel vast distances. The lack of plastic degrading organisms detected after three months of being exposed in five different aquatic locations raises doubts that thebioplastic, PLA, will be biodegraded to any significant extent in aquatic environments. Therefore, government, policy makers and industry leaders should hold bioplastics with the same regard as petroleum-based plastics and work towards solutions that reduce the impact of both petroleum-based plastics and bioplastics on aquatic ecosystems.

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Appendices

Appendix I

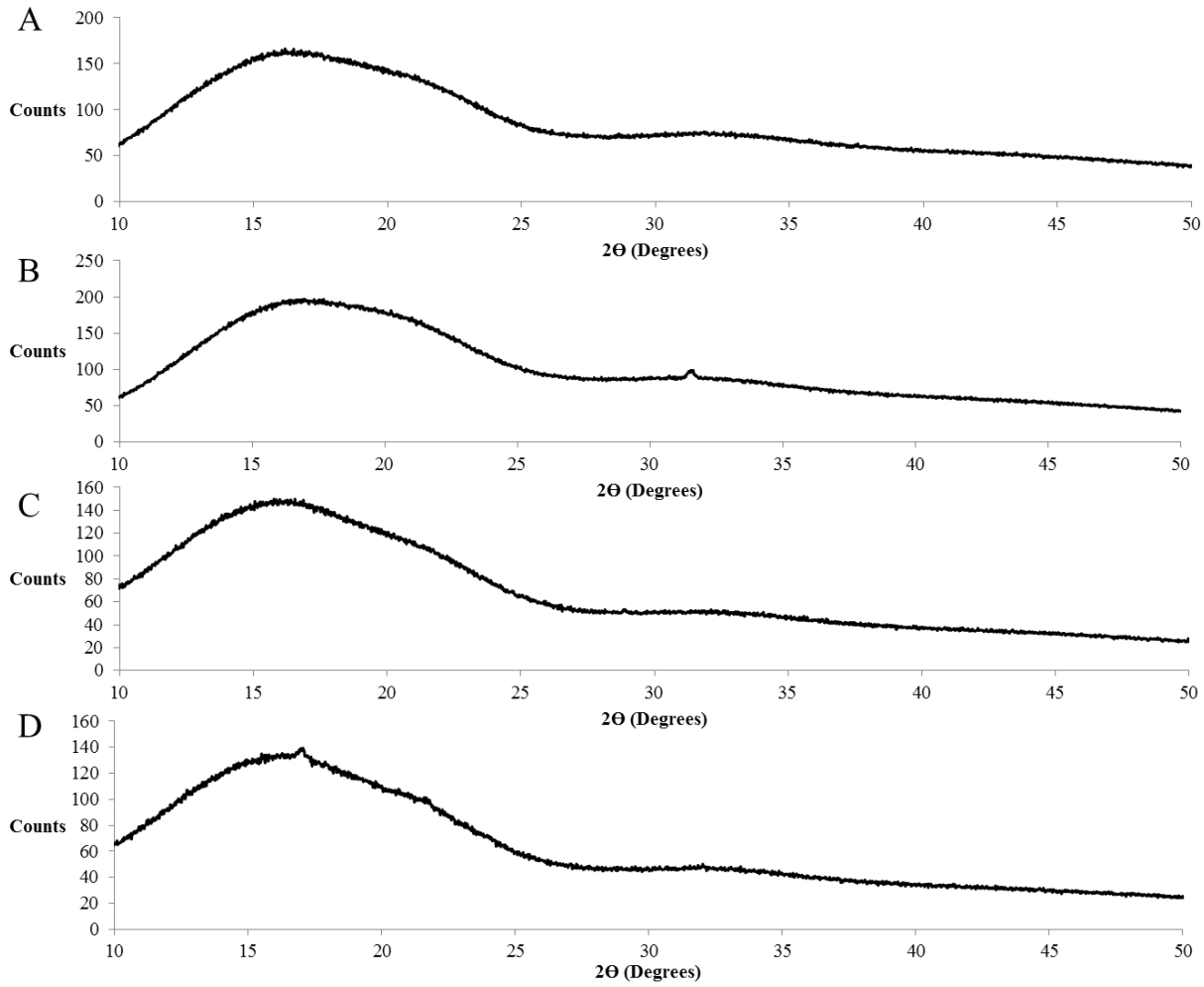

Figure A1. X-ray diffraction spectra of selected polylactic acid coupon samples. A) Day 1, Site 1 (D1S1L) B) Day 1, Site 5 (D1S5L) C) Month 6, Site 1 (M6S1L) D) Month 6, Site 5 (M6S5L).

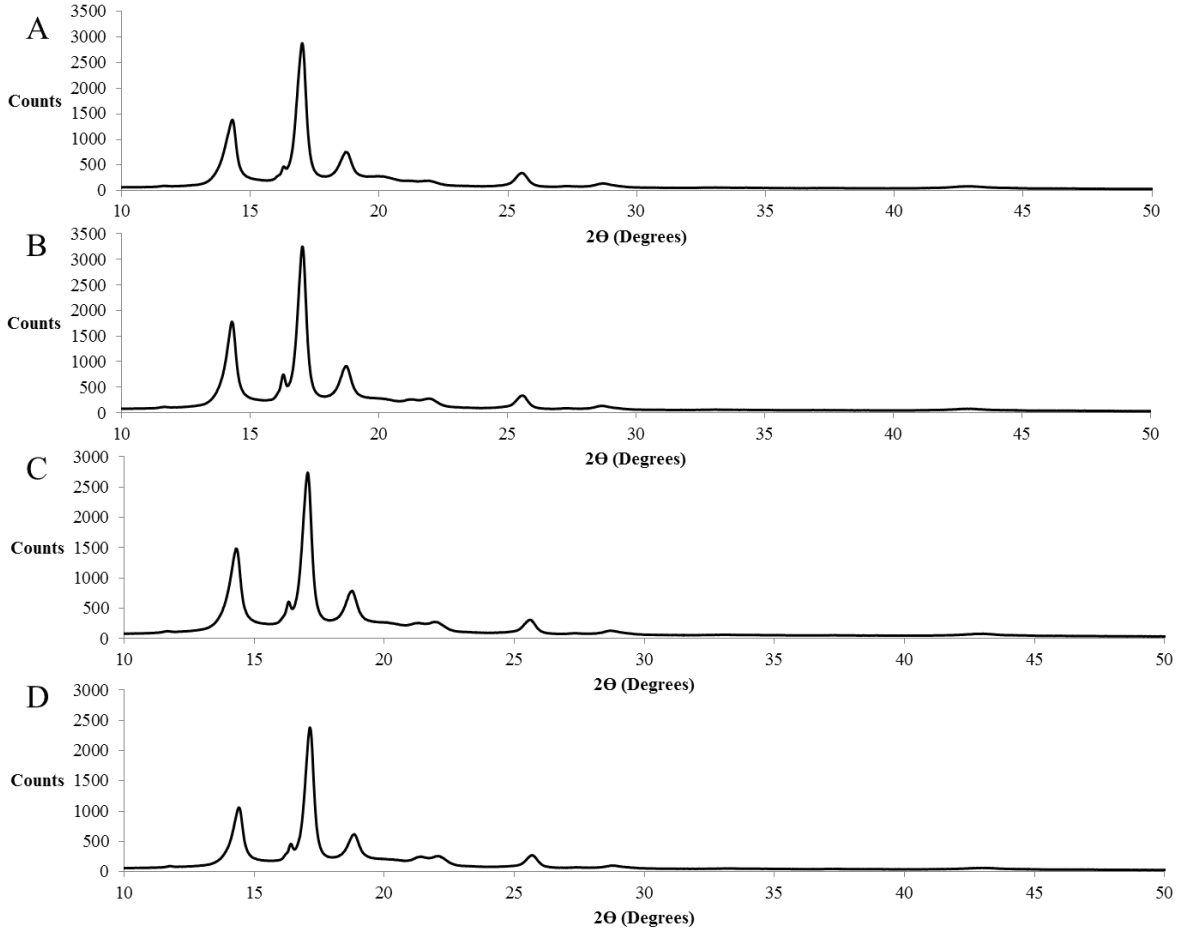

Figure A2. X-ray diffraction spectra of selected polypropylene coupon samples. A) Day 1, Site 1 (D1S1P) B) Day 1, Site 5(D1S5P) C) Month 6, Site 1 (M6S1P) D) Month 6, Site 5 (M6S5P).

Appendix II

Table A1. Estimations of prokaryote observed species (≥97%), Chao1 Richness for species (≥97%), observed OTUs, Chao1 Richness for OTUs and OTU Shannon-Wiener diversity (H'), Simpson diversity (D') and Pielou evenness (J'). Variances are the standard error of the mean.

	Observed Species (97% ID)	**Chao1 Species (97% ID) Richness**	**Observed OTUs**	**Chao1 OTU Richness**	**OTU H'**	**OTU D'**	**OTU J'**
Extraction Blanks	147 ± 33	170 ± 29	420 ± 115	469 ± 122	3.99 ± 0.44	0.9 ± 0.042	0.68 ± 0.04
Site1, Day 7, PP	312 ± 26	345 ± 20	1807 ± 209	2049 ± 213	3.94 ± 0.34	0.91 ± 0.006	0.53 ± 0.04
Site1, Day 7, PLA	243 ± 33	278 ± 25	1155 ± 156	1356 ± 175	3.64 ± 0.41	0.89 ± 0.034	0.52 ± 0.04
Site1, Day 7, Glass	345 ± 60	372 ± 50	1965 ± 388	2118 ± 407	4.28 ± 0.31	0.93 ± 0.016	0.57 ± 0.2
Site1, Day 14, PP*	335	424	1896	2322	4.61	0.95	0.61
Site1, Day 14, PLA	289 ± 15	341 ± 15	1283 ± 187	1578 ± 215	3.7 ± 0.38	0.9 ± 0.028	0.52 ± 0.03
Site1, Day 14, Glass	333 ± 19	379 ± 10	1764 ± 63	2097 ± 26	4.66 ± 0.13	0.95 ± 0.006	0.62 ± 0.01
Site1, Day 28, PP**	435 ± 35	351 ± 113	2920 ± 73	3324 ± 98	3.62 ± 0.05	0.81 ± 0.01	0.45 ± 0.01
Site1, Day 28, PLA**	380 ± 4	424 ± 0.3	2157 ± 65	2543 ± 68	3.26 ± 0.19	0.82 ± 0.002	0.47 ± 0.02
Site1, Day 28, Glass**	277 ± 37	308 ± 33	1288 ± 110	1537 ± 105	2.62 ± 0.23	0.67 ± 0.032	0.37 ± 0.02
Site 1, Month 3, PP	311 ± 5	342 ± 4	2098 ± 26	2414 ± 46	4.47 ± 0.35	0.91 ± 0.03	0.58 ± 0.4
Site 1, Month 3, PLA	309 ± 12	349 ± 10	1953 ± 66	2275 ± 76	4.71 ± 0.19	0.94 ± 0.013	0.62 ± 0.02
Site 1, Month 3, Glass	335 ± 44	374 ± 42	2403 ± 350	2762 ± 404	4.81 ± 0.04	0.93 ± 0.011	0.62 ± 0.01
Site 2, Day 7, PP	481 ± 33	526 ± 25	1754 ± 218	1985 ± 240	3.46 ± 0.25	0.86 ± 0.032	0.46 ± 0.02
Site 2, Day 7, PLA	382 ± 20	428 ± 21	1068 ± 51	1240 ± 50	3.52 ± 0.16	0.88 ± 0.006	0.51 ± 0.02
Site 2, Day 7, Glass	403 ± 32	432 ± 26	1282 ± 125	1427 ± 120	3.39 ± 0.22	0.85 ± 0.031	0.47 ± 0.03

Site 2, Day 14, PP	368 ± 30	415 ± 17	1241 ± 81	1500 ± 70	3.82 ± 0.15	0.92 ± 0.006	0.54 ± 0.01
Site 2, Day 14, PLA*	297	348	1596	2000	3.89	0.91	0.53
Site 2, Day 14, Glass	296 ± 4	337 ± 4	946 ± 42	1179 ± 48	3.09 ± 0.09	0.85 ± 0.011	0.45 ± 0.01
Site 2, Day 28, PP	485 ± 36	523 ± 32	2238 ± 244	2573 ± 252	3.98 ± 0.08	0.91 ± 0.008	0.52 ± 0.01
Site 2, Day 28, PLA	394 ± 78	441 ± 62	1623 ± 425	1885 ± 464	3.29 ± 0.3	0.82 ± 0.047	0.45 ± 0.02
Site 2, Day 28, Glass**	312 ± 70	389 ± 70	1140 ± 195	1466 ± 252	2.57 ± 0.47	0.71 ± 0.106	0.36 ± 0.04
Site 2, Month 3, PP	275 ± 13	330 ± 10	1156 ± 26	1417 ± 59	4.61 ± 0.04	0.97 ± 0.001	0.65 ± 0.01
Site 2, Month 3, PLA	305 ± 14	338 ± 9	1317 ± 127	1536 ± 121	4.43 ± 0.13	0.96 ± 0.005	0.62 ± 0.01
Site 2, Month 3, Glass**	270 ± 22	297 ± 13	1306 ± 77	1561 ± 92	4.76 ± 0.01	0.97 ± 0.01	0.66 ± 0.01
Site 3, Day 7, PP	372 ± 42	416 ± 31	1029 ± 147	1221 ± 160	3.75 ± 0.17	0.92 ± 0.008	0.64 ± 0.01
Site 3, Day 7, PLA**	311 ± 26	359 ± 27	792 ± 38	976 ± 58	3.64 ± 0.09	0.91 ± 0.007	0.55 ± 0.01
Site 3, Day 7, Glass	399 ± 52	454 ± 40	1108 ± 192	1285 ± 181	3.6 ± 0.04	0.9 ± 0.003	0.52 ± 0.01
Site 3, Day 14, PP**	342 ± 26	372 ± 19	1106 ± 142	1282 ± 150	4.42 ± 0.74	0.95 ± 0.024	0.63 ± 0.06
Site 3, Day 14, PLA**	299 ± 13	355 ± 11	692 ± 10	887 ± 22	3.12 ± 0.08	0.88 ± 0.024	0.48 ± 0.01
Site 3, Day 14, Glass	339 ± 33	356 ± 46	1006 ± 115	1187 ± 137	3.27 ± 0.06	0.87 ± 0.011	0.48 ± 0.01
Site 3, Day 28, PP**	424 ± 42	467 ± 37	1627 ± 208	1948 ± 271	4.1 ± 0.09	0.93 ± 0.008	0.56 ± 0.01
Site 3, Day 28, PLA**	364 ± 39	416 ± 27	1164 ± 115	1411 ± 143	3.4 ± 0.17	0.87 ± 0.028	0.48 ± 0.01
Site 3, Day 28, Glass	320 ± 9	353 ± 4	1073 ± 40	1320 ± 30	3.22 ± 0.09	0.88 ± 0.008	0.46 ± 0.01
Site 3, Month 3, PP**	301 ± 20	330 ± 21	1110 ± 4	1263 ± 9	4.51 ± 0.23	0.96 ± 0.017	0.64 ± 0.02
Site 3, Month 3, PLA**	304 ± 22	334 ± 20	1248 ± 104	1448 ± 123	4.81 ± 0.16	0.97 ± 0.004	0.58 ± 0.01
Site 3, Month 3, Glass	330 ± 29	373 ± 29	1297 ± 213	1544 ± 264	4.97 ± 0.29	0.97 ± 0.009	0.7 ± 0.02
Site 4, Day 7, PP	347 ± 33	386 ± 29	1190 ± 15	1369 ± 157	3.27 ± 0.15	0.89 ± 0.011	0.46 ± 0.01
Site 4, Day 7, PLA	258 ± 15	306 ± 19	850 ± 53	1041 ± 61	3.16 ± 0.11	0.89 ± 0.007	0.47 ± 0.01
Site 4, Day 7,	293 ± 34	335 ± 29	924 ± 117	1090 ±	2.97 ±	0.87 ±	0.44 ±

Glass				131	0.09	0.013	0.01
Site 4, Day 14, PP	344 ± 38	384 ± 35	1466 ± 165	1762 ± 207	4.28 ± 0.09	0.95 ± 0.003	0.59 ± 0.01
Site 4, Day 14, Glass	210 ± 34	241 ± 37	737 ± 124	935 ± 162	3.23 ± 0.01	0.9 ± 0.006	0.49 ± 0.01
Site 4, Day 28, PP**	332 ± 23	375 ± 15	865 ± 95	1055 ± 102	3.09 ± 0.14	0.9 ± 0.001	0.46 ± 0.01
Site 4, Day 28, PLA*	405	426	1278	1369	3.77	0.92	0.53
Site 4, Day 28, Glass**	327 ± 14	361 ± 21	966 ± 27	1189 ± 21	4.13 ± 0.01	0.96 ± 0.002	0.6 ± 0.01
Site 4, Month 3, PP	251 ± 15	288 ± 17	1207 ± 54	1403 ± 63	3.9 ± 0.07	0.91 ± 0.004	0.55 ± 0.01
Site 4, Month 3, PLA	259 ± 12	299 ± 7	1181 ± 100	1400 ± 92	4.17 ± 0.35	0.92 ± 0.018	0.59 ± 0.03
Site 4, Month 3, Glass**	331 ± 38	385 ± 35	1788 ± 190	2062 ± 186	4.91 ± 0.01	0.96 ± 0	0.66 ± 0.01
Site 5, Day 7, PP	345 ± 12	372 ± 12	1289 ± 145	1388 ± 157	4.96 ± 0.17	0.97 ± 0.004	0.7 ± 0.01
Site 5, Day 7, PLA	391 ± 21	424 ± 16	1633 ± 111	1804 ± 106	4.76 ± 0.06	0.94 ± 0.004	0.64 ± 0.01
Site 5, Day 14, PP**	344 ± 70	329	1601 ± 225	1874 ± 226	4.46 ± 0.1	0.94 ± 0.006	0.6 ± 0.02
Site 5, Day 14, PLA**	364 ± 72.5	427 ± 43	1697 ± 159	1942 ± 170	4.33 ± 0.1	0.93 ± 0.01	0.58 ± 0.02
Site 5, Day 14, Glass	292 ± 10	329 ± 8	1368 ± 75	1595 ± 88	4.18 ± 0.07	0.94 ± 0.003	0.58 ± 0.01
Site 5, Day 28, PP**	338 ± 46	381 ± 30	1735 ± 271	1967 ± 300	4.58 ± 0.56	0.96 ± 0.001	0.62 ± 0.01
Site 5, Day 28, PLA**	241 ± 63	285 ± 45	1001 ± 236	1181 ± 261	4.14 ± 0.2	0.94 ± 0.007	0.6 ± 0.01
Site 5, Day 28, Glass**	302 ± 24	345 ± 8	1214 ± 2	1415 ± 14	3.88 ± 0.17	0.93 ± 0.007	0.55 ± 0.01
Site 5, Month 3, PP**	327 ± 49	362 ± 36	2311 ± 336	2591 ± 335	5.53 ± 0.27	0.97 ± 0.001	0.72 ± 0.01
Site 5, Month 3, PLA*	250 ±	282	1865	2096	5.81	0.99	0.77
Site 5, Month 3, Glass	310 ± 9	362 ± 12	2204 ± 45	2477 ± 44	5.96 ± 0.02	0.99 ± 0.001	0.77 ± 0.01
Site 1, Day 7, Water**	264 ± 26	316 ± 24	1458 ± 282	1842 ± 321	4.23 ± 0.06	0.96 ± 0.0002	0.59 ± 0.01
Site 1, Day 28, Water*	366	410	1948	2249	4.58	0.96	0.61
Site 2, Day 7, Water	531 ± 46	590 ± 43	2327 ± 420	2784 ± 454	4.99 ± 0.05	0.97 ± 0.0002	0.65 ± 0.01
Site 2, Day 28, Water*	370	441	1778	2110	4.63	0.96	0.62

Site 3, Day 7, Water**	539 ± 30	608 ± 20	1813 ± 145	2202 ± 153	5.18 ± 0.04	0.97 ± 0.001	0.69 ± 0.01
Site 4, Day 7, Water**	208 ± 6	244 ± 53	846 ± 30	1020 ± 34	4.54 ± 0.01	0.97 ± 0.0001	0.67 ± 0.01
Site 4, Day 28, Water*	336	390	1123	1367	4.69	0.97	0.67
Site 5, Day 7, Water*	250	304	1011	1222	4.69	0.97	0.68
Site 5, Day 28, Water*	320	355	1177	1362	3.98	0.91	0.56

*one replicate, **two replicates

Table A2. Estimations of eukaryote observed species (≥97%), Chao1 Richness for species (≥97%), observed OTUs, Chao1 Richness for OTUs and OTU Shannon-Wiener diversity (H'), Simpson diversity (D') and Pielou evenness (J'). Variances are the standard error of the mean.

Sample Group	Observed Species (97% ID)	Chao1 Species (97% ID) Richness	Observed OTUs	Chao1 OTU Richness	OTU H'	OTU D'	OTU J'
Extraction Blanks	124 ± 22	151 ± 31	778 ± 96	1082 ± 183	4.41 ± 0.05	0.96 ± 0.1	0.66 ± 0.01
Site1, Day 7, PP*	172	173	1672	1935	4	1	1
Site1, Day 7, PLA	173 ± 9	201 ± 11	1599 ± 75	1941 ± 74	3.98 ± 0.27	0.92 ± 0.02	0.53 ± 0.03
Site1, Day 7, Glass	168 ± 13	182 ± 16	1811 ± 188	2075 ± 203	4.4 ± 0.13	0.94 ± 0.1	0.58 ± 0.01
Site1, Day 28, PP	190 ± 5	241 ± 5	1861 ± 85	2611 ± 153	3.1 ± 0.12	0.83 ± 0.02	0.41 ± 0.01
Site1, Day 28, PLA	190 ± 10	243 ± 12	2005 ± 235	2893 ± 305	3.2 ± 0.1	0.84 ± 0.1	0.42 ± 0.1
Site1, Day 28, Glass	167 ± 10	206 ± 14	1677 ± 184	2461 ± 248	2.85 ± 0.19	0.82 ± 0.02	0.38 ± 0.02
Site 1, Month 3, PP	178 ± 14	201 ± 18	2243 ± 447	2779 ± 609	4.37 ± 0.17	0.92 ± 0.01	0.57 ± 0.02
Site 1, Month 3, PLA	182 ± 6	218 ± 6	2489 ± 96	3256 ± 68	4.49 ± 0.09	0.94 ± 0.1	0.57 ± 0.1
Site 1, Month 3, Glass	215 ± 8	253 ± 11	3058 ± 50	3831 ± 55	4.12 ± 0.08	0.88 ± 0.01	0.51 ± 0.01
Site 3, Day 7, PP**	159 ± 1	167 ± 0.1	1436 ± 50	1705 ± 36	3.88 ± 0.08	0.91 ± 0.02	0.53 ± 0.01
Site 3, Day 7, PLA**	202 ± 18	219 ± 10	1536 ± 157	1799 ± 177	4.03 ± 0.21	0.9 ± 0.02	0.54 ± 0.02
Site 3, Day 7, Glass	159 ± 15	169 ± 15	1320 ± 126	1558 ± 148	3.25 ± 0.13	0.78 ± 0.02	0.45 ± 0.01
Site 3, Day 28, PP	150 ± 19	171 ± 24	1459 ± 259	1964 ± 389	3.92 ± 0.12	0.92 ± 0.01	0.54 ± 0.02
Site 3, Day 28, PLA	134 ± 5	171 ± 12	1422 ± 122	1893 ± 184	3.61 ± 0.13	0.89 ± 0.01	0.49 ± 0.01
Site 3, Day 28, Glass	130 ± 16	163 ± 22	1291 ± 123	1808 ± 136	3.33 ± 0.03	0.9 ± 0.1	0.46 ± 0.1
Site 3, Month 3, PP	139 ± 20	169 ± 27	1570 ± 314	2048 ± 358	3.9 ± 0.33	0.92 ± 0.03	0.53 ± 0.03
Site 3, Month 3, PLA	110 ± 5	148 ± 7	1231 ± 88	1652 ± 128	3.88 ± 0.24	0.9 ± 0.03	0.54 ± 0.02
Site 3, Month 3, Glass	150 ± 6	180 ± 8	1663 ± 35	2226 ± 36	4.34 ± 0.17	0.95 ± 0.1	0.58 ± 0.02
Site 5, Day 7, PP	172 ± 12	200 ± 15	1813 ± 264	2195 ± 419	4.34 ± 0.08	0.93 ± 0.1	0.58 ± 0.01

Site 5, Day 7, PLA	204 ± 13	222 ± 20	1726 ± 93	1984 ± 97	4.15 ± 0.18	0.92 ± 0.01	0.55 ± 0.02
Site 5, Day 7, Glass	172 ± 17	183 ± 17	1374 ± 175	1537 ± 198	4.54 ± 0.04	0.96 ± 0.1	0.63 ± 0.01
Site 5, Day 28, PP	104 ± 1	171 ± 28	1142 ± 102	1475 ± 134	3.06 ± 0.19	0.83 ± 0.02	0.43 ± 0.02
Site 5, Day 28, PLA	132 ± 20	159 ± 21	1550 ± 251	1939 ± 265	3.57 ± 0.14	0.88 ± 0.02	0.49 ± 0.02
Site 5, Day 28, Glass	140 ± 11	164 ± 10	1376 ± 91	1718 ± 64	3.71 ± 0.09	0.89 ± 0.1	0.51 ± 0.01
Site 5, Month 3, PP	118 ± 6	146 ± 5	1317 ± 182	1705 ± 222	2.57 ± 0.32	0.73 ± 0.06	0.35 ± 0.04
Site 5, Month 3, PLA	100 ± 17	122 ± 22	1092 ± 328	1378 ± 390	2.64 ± 0.35	0.8 ± 0.03	0.38 ± 0.03
Site 5, Month 3, Glass**	148 ± 1	173 ± 0.1	1733 ± 79	2179 ± 93	2.81 ± 0.18	0.74 ± 0.04	0.37 ± 0.02
Site 1, Day 7, Water	213 ± 14	241 ± 6	3700 ± 391	4322 ± 298	5.68 ± 0.04	0.98 ± 0.1	0.69 ± 0.1
Site 1, Day 28, Water	208 ± 7	222 ± 9	2755 ± 189	3054 ± 214	5.2 ± 0.13	0.96 ± 0.1	0.65 ± 0.02
Site 3, Day 7, Water	357 ± 10	386 ± 10	2457 ± 1689	2892 ± 1910	5.12 ± 0.75	0.98 ± 0.1	0.84 ± 0.08
Site 3, Day 28, Water	151 ± 32	175 ± 31	2083 ± 599	2335 ± 666	5.2 ± 0.25	0.95 ± 0.1	0.7 ± 0.1
Site 5, Day 7, Water	234 ± 10	252 ± 13	1681 ± 36	1923 ± 40	5.29 ± 0.01	0.98 ± 0.1	0.71 ± 0.1
Site 5, Day 28, Water	220 ± 19	248 ± 26	1743 ± 218	2080 ± 251	4.7 ± 0.08	0.97 ± 0.1	0.63 ± 0.1

Appendix III

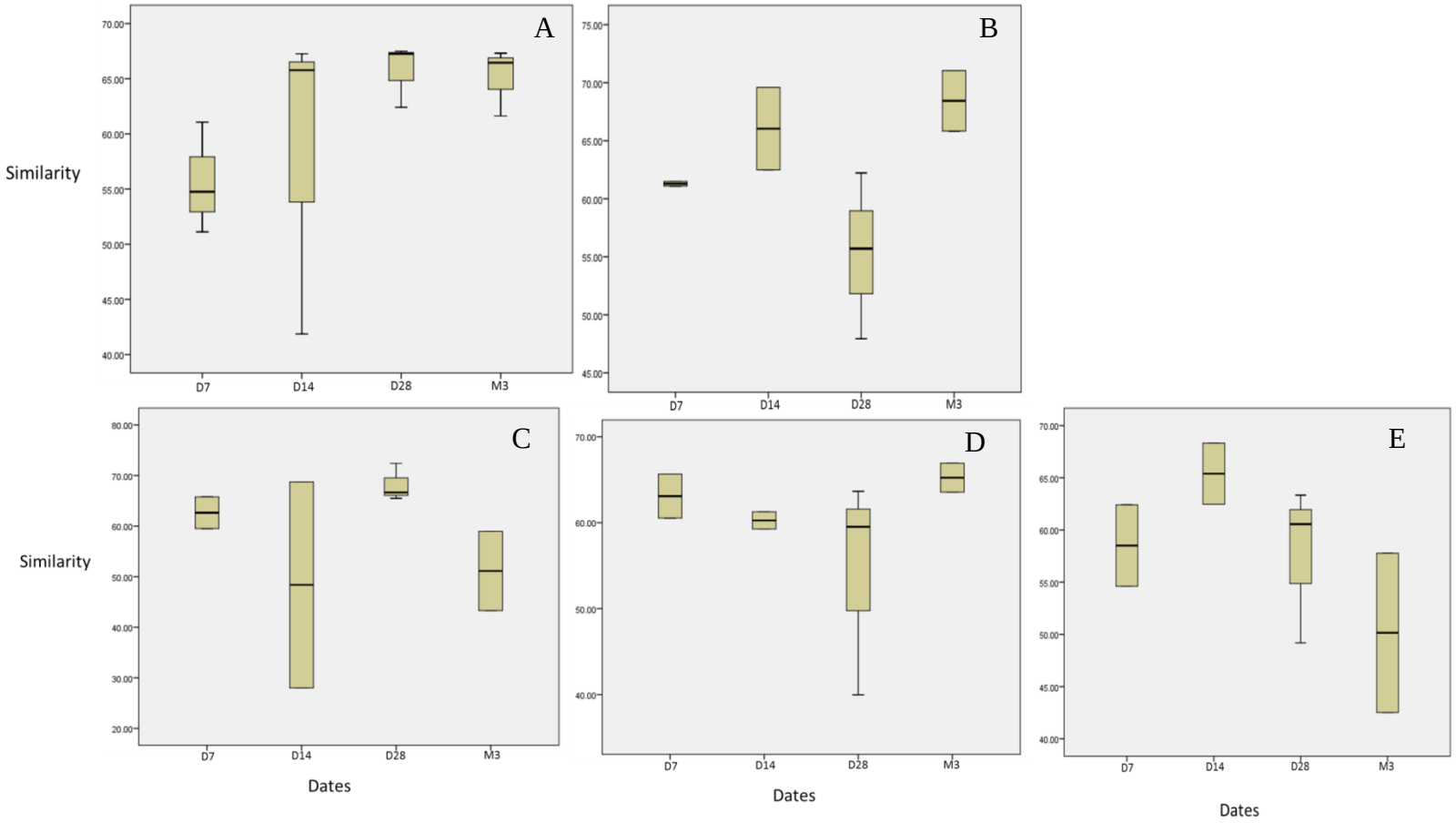

Figure A3. Box-plots of Bray-Curtis community similarity of prokaryotic communities from coupon samples for each of the sampling sites. A) Dights Falls, B) MacRobertson Bridge, C) Federation Square, D) Westgate Bridge, E) Williamstown.

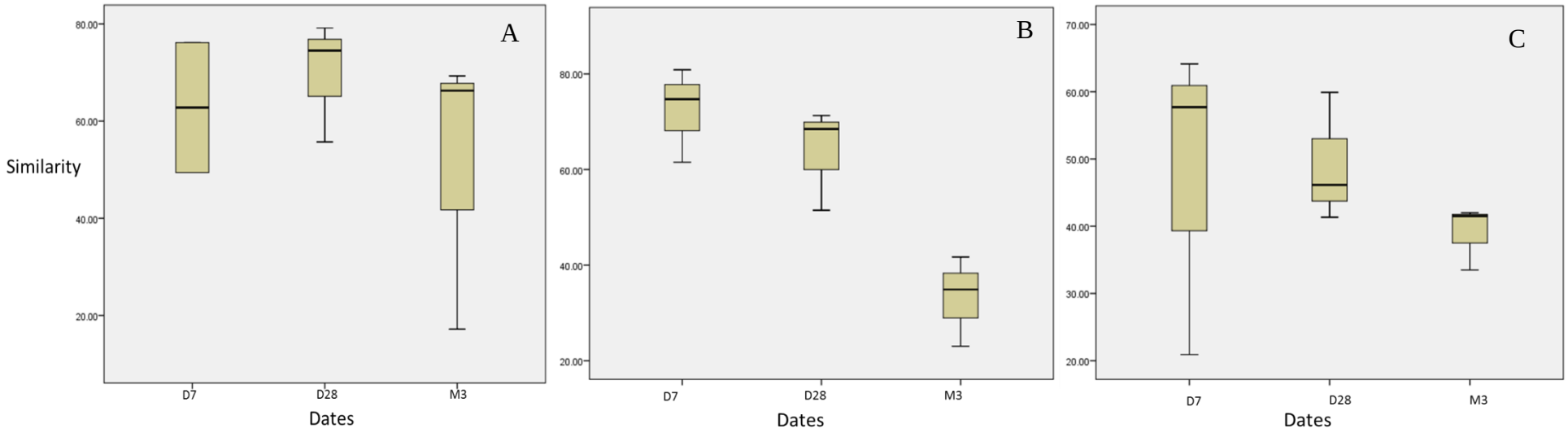

Figure A4. Box-plots of Bray-Curtis community similarity of eukaryotic communities from coupon samples for each of the sampling sites. A) Dights Falls, B) Federation Square, C) Williamstown.

Table A3.Test for Normality of Bray-Curtis similarity for coupon prokaryotic communities, N/A for samples with 2 orless replicates.

		Shapiro-Wilk	
Site	**Date**	**Statistic**	**Significance**
Dights Falls	Day 7	0.977	0.708
	Day 14	0.794	0.100
	Day 28	0.782	0.073
	Month 3	0.860	0.269
MacRobertson Bridge	Day 7	0.830	0.187
	Day 14	N/A	N/A
	Day 28	0.997	0.904
	Month 3	0.997	0.902
Federation Square	Day 7	0.964	0.635
	Day 14	0.927	0.478
	Day 28	0.872	0.302
	Month 3	0.818	0.158
Westgate Bridge	Day 7	0.998	0.916
	Day 14	N/A	N/A
	Day 28	0.876	0.313
	Month 3	1.0	0.993
Williamstown	Day 7	N/A	N/A
	Day 14	0.997	0.893
	Day 28	0.890	0.355
	Month 3	N/A	N/A

Table A4.Test for Normality of Bray-Curtis similarity for coupon eukaryotic communities, N/A for samples with 2 or less replicates.

		Shapiro-Wilk	
Site	**Date**	**Statistic**	**Significance**
Dights Falls	Day 7	N/A	N/A
	Day 28	0.360	0.360
	Month 3	0.099	0.099
Federation Square	Day 7	0.605	0.958
	Day 28	0.252	0.854
	Month 3	0.0703	0.976
Williamstown	Day 7	0.264	0.859
	Day 28	0.482	0.928
	Month 3	0.098	0.793

Appendix IV

Table A5.PERMANOVA and Monte Carlo tests of the Bray-Curtis similarity for each coupon type per each site and time point for the prokaryote samples. Significant results are in bold.

Sites	Groups	t-statistic	P (PERMA-NOVA)	Unique per-mutations	P (Monte Carlo)
Dights Falls	S1D7Glass, S1D7PLA	1.4732	0.086	10	0.132
	S1D7Glass, S1D7PP	1.0867	0.398	10	0.363
	S1D7PLA, S1D7PP	1.327	0.097	10	0.168
	S1D14Glass, S1D14PLA	1.1549	0.087	10	0.275
	S1D14Glass, S1D14PP	1.1257	0.258	4	0.367
	S1D14PLA, S1D14PP	0.7532	0.768	4	0.612
	S1D28Glass, S1D28PLA	1.5321	0.325	3	0.174
	S1D28Glass, S1D28PP	1.9865	0.331	3	0.092
	S1D28PLA, S1D28PP	1.2492	0.331	3	0.308
	S1M3Glass, S1M3PLA	1.1644	0.290	10	0.292
	S1M3Glass, S1M3PP	1.2212	0.099	10	0.200
	S1M3PLA, S1M3PP	1.3835	0.091	10	0.147
MacRobertson Bridge	S2D7Glass, S2D7PLA	1.2027	0.116	10	0.248
	S2D7Glass, S2D7PP	1.1468	0.210	10	0.292
	S2D7PLA, S2D7PP	1.4798	0.121	10	0.108
	S2D14Glass, S2D14PLA	2.402	0.250	4	0.057
	S2D14Glass, S2D14PP	1.4492	0.103	10	0.109
	S2D14PLA, S2D14PP	1.8202	0.256	4	0.098
	S2D28Glass, S2D28PLA	1.0273	0.511	10	0.417
	S2D28Glass, S2D28PP	1.6012	0.095	10	0.121
	S2D28PLA, S2D28PP	0.98922	0.394	10	0.398
	S2M3Glass, S2M3PLA	1.2053	0.186	10	0.297
	S2M3Glass, S2M3PP	1.3861	0.098	10	0.182
	S2M3PLA, S2M3PP	1.4456	0.105	10	0.119
Federation Square	S3D14Glass, S3D14PLA	1.4193	0.098	10	0.180
	S3D14Glass, S3D14PP	1.326	0.096	10	0.217
	S3D28Glass, S3D28PLA	1.3328	0.099	10	0.189
	S3D28Glass, S3D28PP	1.7696	0.097	10	0.087
	S3D7Glass, S3D7PLA	1.1773	0.207	10	0.331
	S3D7Glass, S3D7PP	1.1385	0.282	10	0.287
	S3M3Glass, S3M3PLA	0.85608	0.813	10	0.552

	S3M3Glass, S3M3PP	0.83056	1	10	0.601
	S3D14PLA, S3D14PP	1.0029	0.664	3	0.405
	S3D28PLA, S3D28PP	0.99409	0.664	3	0.480
	S3D7PLA, S3D7PP	1.0905	0.193	10	0.357
	S3M3PLA, S3M3PP	0.98771	0.659	10	0.450
Westgate Bridge	S4D7Glass, S4D7PLA	1.3947	0.085	10	0.154
	S4D7Glass, S4D7PP	1.2872	0.098	10	0.200
	S4D7PLA, S4D7PP	1.4329	0.097	10	0.127
	S4D14Glass, S4D14PLA				
	S4D14Glass, S4D14PP	1.7925	0.099	10	**0.046**
	S4D14PLA, S4D14PP				
	S4D28Glass, S4D28PLA	1.1814	0.296	10	0.285
	S4D28Glass, S4D28PP	1.5456	0.323	3	0.188
	S4D28PLA, S4D28PP	1.3801	0.179	10	0.196
	S4M3Glass, S4M3PLA	1.1991	0.211	10	0.288
	S4M3Glass, S4M3PP	1.5142	0.114	10	0.152
	S4M3PLA, S4M3PP	1.0571	0.316	10	0.369
Williamstown	S5D7Glass, S5D7PLA	N/A	N/A	N/A	N/A
	S5D7Glass, S5D7PLA	N/A	N/A	N/A	N/A
	S5D7PLA, S5D7PP	1.2019	0.096	10	0.25
	S5D14Glass, S5D14PLA	1.323	0.101	10	0.208
	S5D14Glass, S5D14PP	1.4055	0.108	10	0.179
	S5D14PLA, S5D14PP	N/A	N/A	N/A	N/A
	S5D28Glass, S5D28PLA	N/A	N/A	N/A	N/A
	S5D28Glass, S5D28PP	1.59	0.355	3	0.173
	S5D28PLA, S5D28PP	N/A	N/A	N/A	N/A
	S5M3Glass, S5M3PLA	N/A	N/A	N/A	N/A
	S5M3Glass, S5M3PP	1.5199	0.189	10	0.123

Table A6.PERMANOVA and Monte Carlo tests of the Bray-Curtis similarity for each coupon type per each site and time point for the eukaryotic samples.

Sites	Groups	t- statistic	P (PERMANOVA)	Unique permutations	P (Monte Carlo)
Dights Falls	S1D7Glass, S1D7PLA	1.1955	0.111	10	0.255
	S1D7Glass, S1D7PP	1.1256	0.303	3	0.441
	S1D7PLA, S1D7PP	1.1062	0.235	4	0.393
	S1D28Glass, S1D28PLA	1.3285	0.014	56	0.124
	S1D28Glass, S1D28PP	1.1151	0.097	10	0.337
	S1D28PLA, S1D28PP	1.2512	0.116	56	0.177
	S1M3Glass, S1M3PLA	1.245	0.102	10	0.212
	S1M3Glass, S1M3PP	1.0514	0.186	10	0.367
	S1M3PLA, S1M3PP	1.0964	0.097	10	0.326
MacRobertson Bridge	S3D7Glass, S3D7PLA	1.2408	0.088	10	0.258
	S3D7Glass, S3D7PP	1.5757	0.092	10	0.099
	S3D7PLA, S3D7PP	1.3329	0.185	10	0.223
	S3D28Glass, S3D28PLA	1.5617	0.097	10	0.09
	S3D28Glass, S3D28PP	1.3842	0.118	10	0.163
	S3D28PLA, S3D28PP	0.89752	0.737	10	0.527
	S3M3Glass, S3M3PLA	1.0453	0.312	10	0.374
	S3M3Glass, S3M3PP	0.92072	0.906	10	0.473
	S3M3PLA, S3M3PP	0.94961	0.805	10	0.508
Williamstown	S5D7Glass, S5D7PLA	1.3685	0.094	10	0.138
	S5D7Glass, S5D7PP	1.0552	0.204	10	0.357
	S5D7PLA, S5D7PP	1.1447	0.091	10	0.3
	S5D28Glass, S5D28PLA	1.4491	0.096	10	0.122
	S5D28Glass, S5D28PP	1.445	0.098	10	0.125
	S5D28PLA, S5D28PP	1.1206	0.18	10	0.351
	S5M3Glass, S5M3PLA	0.98071	0.614	10	0.448
	S5M3Glass, S5M3PP	1.0883	0.319	10	0.376

Appendix V

Table A7.List of pathogenic bacteria screened for in the bacterial biofilm and planktonic communities.

Pathogenic Species	Genus Present	Species (97% ID) Present
Abiotrophia adiacens (Streptococcus adiacens)	No	No
Abiotrophia defectiva (Streptococcus defectiva)	No	No
Acetivibrio ethanologignens	No	No
Acholeplasma axanthum	No	No
Acholeplasma granularm	No	No
Acholeplasma hippikon	No	No
Acholeplasma laidlawii	No	No
Acholeplasma modicum	No	No
Acholeplasma multilocale	No	No
Acidaminococcus fermentans	Yes	yes
Acinetobacter baumannii	Yes	No
Acinetobacter calcoaceticus	Yes	Yes
Acinetobacter haemolyticus	Yes	No
Acinetobacter johnsonii	Yes	Yes
Acinetobacter junii	Yes	No
Acinetobacter lwoffii	Yes	Yes
Actinobacillus capsulatus	No	No
Actinobacillus delphincola	No	No
Actinobacillus equuli	No	No
Actinobacillus hominis	No	No
Actinobacillus lignieresii	No	No
Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)	No	No
Actinobacillus rossii	No	No
Actinobacillus seminis	No	No
Actinobacillus suis	No	No
Actinobacillus ureae (Pasteurella ureae)	No	No
Actinomadura malachitica (Actinomadura viridis)	No	No
Actinomadura polychroma (Microtetraspora polychroma)	No	No
Actinomyces bernardiae	Yes	No
Actinomyces bovis	Yes	No

Actinomyces gerencseriae (Actinomyces israelii)	Yes	No
Actinomyces hordeovulneris	Yes	No
Actinomyces hyovaginalis	Yes	No
Actinomyces israelii	Yes	No
Actinomyces meyeri (Actinobacterium meyeri)	Yes	No
Actinomyces naeslundii	Yes	No
Actinomyces neuii	Yes	No
Actinomyces odontolyticus	Yes	No
Actinomyces pyogenes (Corynebacterium pyogenes)	Yes	No
Actinomyces radingae	Yes	No
Actinomyces suis (Eubacterium suis)	Yes	No
Actinomyces turicensis	Yes	No
Actinomyces viscosus	Yes	No
Aegyptianella pullorum	No	No
Aerococcus urinae	No	No
Aerococcus viridans	No	No
Aeromonas allosaccharophila	No	No
Aeromonas caviae	No	No
Aeromonas enteropelogenes	No	No
Aeromonas hydrophila	No	No
Aeromonas ichthiosmia	No	No
Aeromonas jandaei	No	No
Aeromonas punctata	No	No
Aeromonas salmonicida	No	No
Aeromonas schubertii	No	No
Aeromonas sorbia	No	No
Aeromonas trota	No	No
Afipia broomeae	No	No
Afipia clevelandensis	No	No
Afipia felis	No	No
Alcaligenes faecalis	No	No
Alcaligenes piechaudii	No	No
Alcaligenes xylosoxydans	No	No
Alloiococcus otitis	No	No
Allomonas enterica	No	No
Anaerorhabdus furcosus	No	No
Anaplasma caudatum	No	No
Anaplasma centrale	No	No
Anaplasma marginale	No	No
Anaplasma ovis	No	No
Arcabobacterium haemolyticum	No	No

Arcobacter butzleri (Camplyobacter butzleri)	Yes	No
Arcobacter cryaerophilus (Camplyobacter cryaerophila)	Yes	No
Atopobium minutum (Lactobacillus minutus)	No	No
Atopobium parvulum (Streptococcus parvulus)	No	No
Atopobium rimae (Lactobacillus rimae)	No	No
Bacillus anthracis	Yes	No
Bacillus cereus	Yes	Yes
Bacteroides caccae	Yes	No
Bacteroides capillosus	Yes	No
Bacteroides cellulosolvens	Yes	No
Bacteroides distasonis	Yes	No
Bacteroides eggerthii	Yes	No
Bacteroides forsythus	Yes	No
Bacteroides fragilis	Yes	No
Bacteroides helcogenes	Yes	No
Bacteroides pectionphilus	Yes	No
Bacteroides putredinis	Yes	No
Bacteroides pyogenes	Yes	No
Bacteroides splanchnicus	Yes	No
Bacteroides stercoris	Yes	No
Bacteroides suis	Yes	No
Bacteroides tectum	Yes	No
Bacteroides thetaiotaomicron	Yes	No
Bacteroides uniformis	Yes	No
Bacteroides ureolyticus	Yes	No
Balneatrix alpica	No	No
Bartonella bacilliformis	No	No
Bartonella doshiae	No	No
Bartonella elziabethae (Rochalimaea elziabethae)	No	No
Bartonella grahamii	No	No
Bartonella henselae (Rochalimaea henselae)	No	No
Bartonella peromysci (Grahamella peromysci)	No	No
Bartonella quintana (Rochalimaea quintana)	No	No
Bartonella talpae (Grahamella talpae)	No	No
Bartonella taylorii	No	No
Bartonella vinsonii	No	No

Beneckea alginolytica (Vibrio alginolyticus)	No	No
Beneckea pelagia (Listonella pelagia)	No	No
Bergeyella zoohelcum (Weeksella zoohelcum)	No	No
Bifidobacterium dentium	Yes	No
Bilophila wadsworthia	No	No
Bordetella avium	Yes	No
Bordetella bronchinseptica	Yes	No
Bordetella hinzii	Yes	No
Bordetella holmesii	Yes	No
Bordetella parapertussis	Yes	No
Bordetella pertussis	Yes	No
Borrelia afzelii	No	No
Borrelia anserina	No	No
Borrelia baltazardii	No	No
Borrelia brasiliensis	No	No
Borrelia burgdorferi	No	No
Borrelia caucasica	No	No
Borrelia coriaceae	No	No
Borrelia crocidurae	No	No
Borrelia dugesii	No	No
Borrelia duttonii	No	No
Borrelia garinii	No	No
Borrelia graingeri	No	No
Borrelia harveyi	No	No
Borrelia hermsii	No	No
Borrelia hispanica	No	No
Borrelia latyschewii	No	No
Borrelia mazzottii	No	No
Borrelia parkeri	No	No
Borrelia persica	No	No
Borrelia recurrentis	No	No
Borrelia theileri	No	No
Borrelia tillae	No	No
Borrelia turicatae	No	No
Borrelia venezuelensis	No	No
Brachyspria aalborgi	No	No
Brevibacterium mcbrellneri	Yes	No
Brevinema andersonii	No	No
Brevundimonas diminuta	Yes	No
Brucella neotomae	No	No
Burkholderia cepacia (Pseudomonas caryophylli)	Yes	No

Burkholderia mallei (Pseudomonas mallei)	Yes	No
Burkholderia pseudomallei (Pseudomonas pseudomallei)	Yes	No
Burkholderia vietnamiensis	Yes	No
Calymmatobacterium granlomatis	No	No
Camplyobacter butzleri (Arcobacter butzleri)	Yes	No
Camplyobacter coli	Yes	No
Camplyobacter concisus	Yes	No
Camplyobacter curvus (Wolinella curva)	Yes	No
Camplyobacter fetus	Yes	No
Camplyobacter gracilis	Yes	No
Camplyobacter hyoilei	Yes	No
Camplyobacter hyointestinalis	Yes	No
Camplyobacter jejuni	Yes	Yes
Camplyobacter mucosalis	Yes	No
Camplyobacter rectus (Wolinella recta)	Yes	No
Camplyobacter sputorum	Yes	No
Camplyobacter upsaliensis	Yes	No
Capnocytophaga canimorsus	Yes	No
Capnocytophaga cynodegmi	Yes	No
Capnocytophaga ginigivalis	Yes	No
Capnocytophaga granuloas	Yes	No
Capnocytophaga haemolytica	Yes	No
Capnocytophaga orchracea (Bacteroides ochraceus)	Yes	No
Capnocytophaga sputigena	Yes	No
Cardiobacterium hominis	No	No
Catonella morbi	No	No
Cedecea davisae	No	No
Cedecea lapagei	No	No
Cedecea neteri	No	No
Centipeda periodontii	No	No
Chlamydia pecorum	No	No
Chlamydia pneumoniae	No	No
Chlamydia psittaci	No	No
Chlamydia trachomatis	No	No
Chromobacterium violaceum	No	No
Chryseobacterium gleum (Flavobacterium gleum)	Yes	No
Chryseobacterium indologenes (Flavobacterium indologenes)	Yes	No
Chryseobacterium	Yes	No

meningosepticum (Flavobacterium meningosepticum)		
Chryseobacterium scophthalmum (Flavobacterium scophthalmum)	Yes	No
Chryseomonas luteola (Pseudomonas luteola)	No	No
Chryseomonas polytricha	No	No
Citrobacter amalonaticus (Levinea amalonatica)	No	No
Citrobacter brakii	No	No
Citrobacter farmeri	No	No
Citrobacter freundii	No	No
Citrobacter koseri	No	No
Citrobacter rodentium	No	No
Citrobacter sedlakii	No	No
Citrobacter werkmanii	No	No
Citrobacter youngae	No	No
Clostridium absonum	Yes	No
Clostridium barati	Yes	No
Clostridium bifermentans	Yes	No
Clostridium botulinum	Yes	No
Clostridium cadaveris	Yes	No
Clostridium chauvoei	Yes	No
Clostridium clostridiiforme	Yes	No
Clostridium colinum	Yes	No
Clostridium difficile	Yes	Yes
Clostridium felsineum	Yes	No
Clostridium ghoni	Yes	No
Clostridium glycolicum	Yes	No
Clostridium haemonlyticum	Yes	No
Clostridium hastiforme	Yes	No
Clostridium histolyticum	Yes	No
Clostridium indolis	Yes	No
Clostridium innocuum	Yes	No
Clostridium leptum	Yes	No
Clostridium magnum	Yes	Yes
Clostridium nexile	Yes	No
Clostridium oroticum	Yes	No
Clostridium paraputrificum	Yes	No
Clostridium perfringens	Yes	Yes
Clostridium piliforme	Yes	No
Clostridium putrificum	Yes	No
Clostridium ramosum	Yes	No
Clostridium septicum	Yes	No
Clostridium sordellii	Yes	No

Clostridium sphenoides	Yes	No
Clostridium sporogenes	Yes	No
Clostridium subterminale	Yes	No
Clostridium symbiosum	Yes	No
Clostridium tertium	Yes	No
Clostridium tetani	Yes	No
Comamonas terrigena (Aquaspirillum aquatcum)	No	No
Corynebacterium accolens	Yes	No
Corynebacterium afermentans	Yes	No
Corynebacterium argentoratense	Yes	No
Corynebacterium auris	Yes	No
Corynebacterium bovis	Yes	No
Corynebacterium callunae	Yes	No
Corynebacterium diphtheriae	Yes	No
Corynebacterium equi	Yes	No
Corynebacterium hoagii	Yes	No
Corynebacterium kjeikeium	Yes	No
Corynebacterium matruchotti (Bacterionema matruchotti)	Yes	No
Corynebacterium minutissimum	Yes	No
Corynebacterium mycetoides	Yes	No
Corynebacterium pilosum	Yes	No
Corynebacterium propinquum	Yes	No
Corynebacterium pseudodiphtheriticum	Yes	No
Corynebacterium pseudotuberculosis	Yes	No
Corynebacterium renale	Yes	No
Corynebacterium seminale	Yes	No
Corynebacterium striatum	Yes	No
Corynebacterium ulcerans	Yes	No
Corynebacterium urealyticum	Yes	No
Corynebacterium xerosis	Yes	No
Cowdria ruminantium	No	No
Coxiella burnetii	No	No
Cytophaga diffluens	Yes	No
Cytophaga saccharophila (Flavobacterium saccharophilum)	Yes	No
Dermatophilus chelonae	No	No
Dermatophilus congolensis	No	No
Dialister pneumosintes (Bacteroides pneumosintes)	Yes	No
Dichelobacter nodosus (Bacteroides nodosus)	No	No
Edwardsiella anguillimortifera	No	No

Edwardsiella ictaluri	No	No
Edwardsiella tarda	No	No
Ehrlichia canis	No	No
Ehrlichia chaffeensis	No	No
Ehrlichia equi	No	No
Ehrlichia ewingii	No	No
Ehrlichia muris	No	No
Ehrlichia phagocytophila	No	No
Ehrlichia sennetsu (Rickettsia sennetsu)	No	No
Eikenella corrodens	No	No
Empedobacter brevis (Flavobacterium breve)	No	No
Enterobacter aerogenes	No	No
Enterobacter amnigenus	No	No
Enterobacter asburiae	No	No
Enterobacter cancerogenus (Erwinia cancerogena)	No	No
Enterobacter cloacae	No	No
Enterobacter gergoviae	No	No
Enterobacter hormaechei	No	No
Enterobacter intermedius	No	No
Enterobacter taylorae	No	No
Enterococcus avium	No	No
Enterococcus dispar	No	No
Enterococcus durans (Streptococcus durans)	No	No
Enterococcus faecalis (Streptococcus faecalis)	No	No
Enterococcus faecium (Streptococcus faecium)	No	No
Enterococcus flavescens	No	No
Enterococcus gallinarum (Streptococcus gallinarum)	No	No
Enterococcus hirae	No	No
Enterococcus psuedoavium	No	No
Enterococcus raffinosus	No	No
Enterococcus solitarius	No	No
Eperythrozoon coccoides	No	No
Eperythrozoon ovis	No	No
Eperythrozoon parvum	No	No
Eperythrozoon suis	No	No
Eperythrozoon wenyonii	No	No
Erysipelothrix tonsillarum	No	No
Escherichia colinum	Yes	No
Escherichia fergusonii	Yes	No

Escherichia hermannii	Yes	No
Escherichia vuleris	Yes	No
Eubacterium aerofaciens	Yes	No
Eubacterium alactolyticum	Yes	No
Eubacterium brachy	Yes	No
Eubacterium combesii	Yes	No
Eubacterium contortum	Yes	No
Eubacterium formicigenerans	Yes	No
Eubacterium lentum	Yes	No
Eubacterium limosum	Yes	No
Eubacterium minutum	Yes	No
Eubacterium moniliforme	Yes	No
Eubacterium nitriogenes	Yes	No
Eubacterium nodatum	Yes	No
Eubacterium saphenum	Yes	No
Eubacterium tarantellus	Yes	No
Eubacterium tenue	Yes	No
Eubacterium timidium	Yes	No
Eubacterium tortuosum	Yes	No
Eubacterium ventriosum	Yes	No
Eubacterium yurii	Yes	No
Egwingella americana	No	No
Falcivibrio grandis	No	No
Falcivibrio vaginalis	No	No
Flavimonas oryzihabitans (Pseudomonas oryzihabitans)	No	No
Flavobacterium branchiophilum	Yes	No
Flavobacterium columnare (Flexibacter columnaris)	Yes	No
Flavobacterium hydatis (Cytophaga aquatilis)	Yes	No
Flavobacterium johnsoniae (Cytophaga johnsonae)	Yes	No
Flavobacterium mizutaii (Sphingobacterium mizutae)	Yes	No
Flavobacterium odoratum	Yes	No
Flavobacterium psychrophilum (Flexibacter pyschrophilus)	Yes	Yes
Flexibacter maritimus	No	No
Flexibacter ovolyticus	No	No
Fluoribacter bozemanae (Legionella bozemanii)	No	No
Fluoribacter dumoffi (Legionella dumoffii)	No	No
Fluoribacter gormanii (Legionella gormanii)	No	No

Francisella novicida	Yes	No
Francisella philomiragia (Yersinia philomiragia)	Yes	No
Francisella tularensis	Yes	No
Fusibacterium alocis	Yes	No
Fusibacterium gonidiaformans	Yes	No
Fusibacterium mortiferum	Yes	Yes
Fusibacterium naviforme	Yes	No
Fusibacterium necrogenes	Yes	No
Fusibacterium necrophorum	Yes	No
Fusibacterium nucleatum	Yes	Yes
Fusibacterium periodonticum	Yes	Yes
Fusibacterium prausnitzii	Yes	No
Fusibacterium russi	Yes	No
Fusibacterium sulci	Yes	No
Fusibacterium ulcerans	Yes	No
Fusibacterium varium	Yes	No
Garnerella vaginalis (Haemophilus vaginalis)	No	No
Germella haemolysans	No	No
Germella morbillorum (Streptococcus morbillorum)	No	No
Globicatella sanguis	No	No
Gordona aichiensis (Rhodococcus aichiensis)	No	No
Gordona bronchialis (Rhodococcus bronchialis)	No	No
Gordona sputi (Rhodococcus sputi)	No	No
Haemobartonella canis	No	No
Haemobartonella felis	No	No
Haemobartonella muris	No	No
Haemophilus actinomycetemcomitans (Actinobacillus actinomycetemcomitans)	No	No
Haemophilus aeqyptius	No	No
Haemophilus aphrophilus	No	No
Haemophilus ducreyi	No	No
Haemophilus haemoglobinophilus	No	No
Haemophilus influenzae	No	No
Haemophilus paracuniculus	No	No
Haemophilus paragallinarum	No	No
Haemophilus parahaemolyticus	No	No
Haemophilus parainfluenae	No	No
Haemophilus paraphrohaemolyticus	No	No

Haemophilus paraphrophilus	No	No
Haemophilus parasuis	No	No
Haemophilus sengnis	No	No
Hafnia alvei	No	No
Hallella seregens	No	No
Helcococcus kunzii	No	No
Helicobacter acinonyx	No	No
Helicobacter cinaedi (Campylobacter cinaedi)	No	No
Helicobacter fennelliae (Campylobacter fennelliae)	No	No
Helicobacter hepaticus	No	No
Helicobacter mustelae (Campylobacter mustelae)	No	No
Helicobacter pullorum	No	No
Helicobacter pylori (Campylobacter pylori)	No	No
Johnsonella ignava	No	No
Jonesia denitrificans (Listeria denitrificans)	No	No
Kingella denitrificans	No	No
Kingella kingae	No	No
Kingella oralis	No	No
Klebsiella mobilis	No	No
Klebsiella ornithinolytica	No	No
Klebsiella oxytoca	No	No
Klebsiella pneumoniae	No	No
Kluyvera ascorbata	No	No
Kluyvera cryocrescens	No	No
Korserlla trabulsii	No	No
Lactobacillus catenaformis	Yes	No
Lactobacillus rhamnosus	Yes	No
Lactobacillus vaccinostercus	Yes	No
Lactococcus garvieae (Streptococcus garvieae)	No	No
Lawsonia intracellularis	No	No
Leclercia adecarboxylata (Escherichia adecarboxylata)	No	No
Legionella anisa	Yes	No
Legionella birminghamensis	Yes	No
Legionella cincinnatiensis	Yes	No
Legionella geestiana	Yes	No
Legionella hackeliae	Yes	No
Legionella jordanis	Yes	No
Legionella lansingensis	Yes	No
Legionella longbeachae	Yes	Yes

Legionella maceachernii	Yes	No
Legionella parisiensis	Yes	No
Legionella pneumophila	Yes	No
Legionella sainthelensi	Yes	No
Legionella tucsonensis	Yes	No
Legionella wadsworthii	Yes	No
Leptospira borgpetersenii	No	No
Leptospira inadai	No	No
Leptospira interrogans	No	No
Leptospira kirschneri	No	No
Leptospira noguchii	No	No
Leptospira santarosai	No	No
Leptospira weilii	No	No
Levinea malonatica	No	No
Levinea ivanoii	No	No
Levinea monocytogenes	No	No
Listonella anguillarum	Yes	Yes
Megasphaera elsdenii	Yes	No
Mitsuokella multiacidus (Bacteroides multiacidus)	Yes	No
Mobiluncus curtisii	No	No
Mobiluncus mulieris	No	No
Moellerella wisconsensis	No	No
Moraxella euqi	No	No
Moraxella saccharolytica	No	No
Moraxella catarrhalis	No	No
Moraxella ovis	No	No
Moraxella lacunata	No	No
Moraxella nonliquefaciens	No	No
Moraxella osloensis	No	No
Moraxella phenylpyrvica	No	No
Morganell morganii	No	No
Morococcus cerebrosus	No	No
Mycobacterium abscessus	Yes	No
Mycobacterium aurun	Yes	No
Mycobacterium avium	Yes	No
Mycobacterium bovis	Yes	No
Mycobacterium branderi	Yes	No
Mycobacterium celatum	Yes	No
Mycobacterium chelonae	Yes	No
Mycobacterium conspicuum	Yes	No
Mycobacterium farcinogenes	Yes	No
Mycobacterium flavescens	Yes	No
Mycobacterium fortuitum	Yes	No

Mycobacterium gasti	Yes	No
Mycobacterium genavense	Yes	No
Mycobacterium haemophilum	Yes	No
Mycobacterium interjectum	Yes	No
Mycobacterium intermedium	Yes	No
Mycobacterium intracellulare	Yes	No
Mycobacterium kansasii	Yes	No
Mycobacterium leprae	Yes	No
Mycobacterium lepraemurium	Yes	No
Mycobacterium malmoense	Yes	No
Mycobacterium marinum	Yes	No
Mycobacterium microti	Yes	No
Mycobacterium mucogenicum	Yes	No
Mycobacterium porcinum	Yes	No
Mycobacterium scrofulaceum	Yes	No
Mycobacterium senegalense	Yes	No
Mycobacterium shimoidei	Yes	No
Mycobacterium simiae	Yes	No
Mycobacterium szulgai	Yes	No
Mycobacterium tuberculosis	Yes	No
Mycobacterium ulcerans	Yes	No
Mycobacterium vaccae	Yes	No
Mycobacterium xenopi	Yes	No
Mycoplasma adleri	Yes	No
Mycoplasma agalactiae	Yes	No
Mycoplasma alkalscens	Yes	No
Mycoplasma anatis	Yes	No
Mycoplasma arginini	Yes	No
Mycoplasma arthritdis	Yes	No
Mycoplasma bovigenitalium	Yes	No
Mycoplasma bovirhinis	Yes	No
Mycoplasma bovis	Yes	No
Mycoplasma bovoculi	Yes	No
Mycoplasma buteonis	Yes	No
Mycoplasma californicum	Yes	No
Mycoplasma canadense	Yes	No
Mycoplasma canis	Yes	No
Mycoplasma capricolum	Yes	No
Mycoplasma collis	Yes	No
Mycoplasma columbinasale	Yes	No
Mycoplasma conjunctivae	Yes	No
Mycoplasma corogypsi	Yes	No
Mycoplasma cynos	Yes	No
Mycoplasma dispar	Yes	No

Mycoplasma edwardii	Yes	No
Mycoplasma equigenitalium	Yes	No
Mycoplasma equirhinis	Yes	No
Mycoplasma falconis	Yes	No
Mycoplasma felis	Yes	No
Mycoplasma fermentans	Yes	No
Mycoplasma flocculare	Yes	No
Mycoplasma gallinarum	Yes	No
Mycoplasma gallisepticum	Yes	No
Mycoplasma gallopavonis	Yes	No
Mycoplasma gateae	Yes	No
Mycoplasma genitalium	Yes	No
Mycoplasma glycophilum	Yes	No
Mycoplasma gypis	Yes	No
Mycoplasma hominis	Yes	No
Mycoplasma hyopneumoniae	Yes	No
Mycoplasma hyorhinis	Yes	No
Mycoplasma hyosynoviae	Yes	No
Mycoplasma imitans	Yes	No
Mycoplasma iowae	Yes	No
Mycoplasma lipofaciens	Yes	No
Mycoplasma maculosum	Yes	No
Mycoplasma meleagridis	Yes	No
Mycoplasma mycoides	Yes	No
Mycoplasma neurolyticum	Yes	No
Mycoplasma ovipneumoniae	Yes	No
Mycoplasma pernetrans	Yes	No
Mycoplasma phocacerebrale	Yes	Yes
Mycoplasma phocarhinis	Yes	No
Mycoplasma phocidae	Yes	No
Mycoplasma pneumoniae	Yes	No
Mycoplasma pulmonis	Yes	No
Mycoplasma putrefaciens	Yes	No
Mycoplasma salivarium	Yes	No
Mycoplasma spumans	Yes	No
Mycoplasma sturni	Yes	No
Mycoplasma siuipneumoniae	Yes	No
Mycoplasma synoviae	Yes	No
Mycoplasma verecundum	Yes	Yes
Neisseria elongata	Yes	No
Neisseria flavescens	Yes	No
Neisseria gonorrhoeae	Yes	No
Neisseria iguanae	Yes	No
Neisseria lactmica	Yes	No

Neisseria meningitdis	Yes	No
Neisseria mucosa	Yes	No
Neisseria ovis	Yes	No
Neisseria sicca	Yes	No
Neisseria subflava	Yes	No
Neisseria weaveri	Yes	Yes
Neorickettsia helminthoeca	No	No
Nocardia asteroides	Yes	No
Nocardia brasiliensis	Yes	No
Nocardia farcinica	Yes	No
Nocardia nova	Yes	No
Nocardia otitidiscaviarum	Yes	No
Nocardia pseudobrasiliensis	Yes	No
Nocardia seriolae	Yes	No
Nocardia transvalensis	Yes	No
Nocardiopsis dassonvillei	No	No
Ochrobactrum anthropi	No	No
Orientia tsutsugamushi	No	No
Ornithobacterium rhinotracheale	No	No
Pantoea agglomerans (Enterbacter agglomerans)	No	No
Pasteurella bettyae (Pasteurella betti)	No	No
Pasteurella caballi	No	No
Pasteurella canis	No	No
Pasteurella dagmatis	No	No
Pasteurella galinarum	No	No
Pasteurella haemolytica	No	No
Pasteurella lymphangitidis	No	No
Pasteurella mairii	No	No
Pasteurella multocida	No	No
Pasteurella pneumotropica	No	No
Pasteurella stomatis	No	No
Pasteurella testudinis	No	No
Pasteurella trehalosi	No	No
Peptococcus niger	Yes	No
Peptostreptococcus anaerobius	Yes	No
Peptostreptococcus asaccharolyticus (Peptococcus asaccharolyticus)	Yes	No
Peptostreptococcus indolicus (Peptococcus indolicus)	Yes	No
Peptostreptococcus lacrimalis	Yes	No
Peptostreptococcus magnus (Peptococcus magnus)	Yes	No

Peptostreptococcus micros	Yes	No
Peptostreptococcus prevotii (Peptococcus prevotii)	Yes	No
Peptostreptococcus vaginalis	Yes	No
Photobacterium damsela (Listonella damsela)	Yes	Yes
Piscirickettsia salmonis	No	No
Plesionmonas vacuolata	No	No
Porphyromonas asaccharolytica (Bacteroides asaccharolyticus)	Yes	No
Porphyromonas cangingivalis	Yes	No
Porphyromonas canoris	Yes	No
Porphyromonas cansulci	Yes	Yes
Porphyromonas catoniae (Oribaculum catoniae)	Yes	No
Porphyromonas circumdentaria	Yes	No
Porphyromonas endodontalis (Bacteroides endodontalis)	Yes	No
Porphyromonas gingivalis (Bacteroides gingivalis)	Yes	No
Porphyromonas macacae (Bacteroides macacae)	Yes	No
Porphyromonas salivosa (Bacteroides salivosus)	Yes	No
Prevotella bivia (Bacteroides bivius)	Yes	No
Prevotella buccae (Bacteroides buccae)	Yes	No
Prevotella buccalis (Bacteroides buccalis)	Yes	Yes
Prevotella corporis (Bacteroides corporis)	Yes	No
Prevotella dentalis (Mitsuokella dentalis)	Yes	No
Prevotella denticola (Bacteroides denticola)	Yes	No
Prevotella disiens (Bacteroides disiens)	Yes	No
Prevotella intermedia (Bacteroides intermedius)	Yes	No
Prevotella loescheii (Bacteroides loescheii)	Yes	No
Prevotella melaninogenica (Bacteroides melaninogenicus)	Yes	No
Prevotella nigrescens	Yes	No
Prevotella oralis (Bacteroides oralis)	Yes	No
Prevotella oris (Bacteroides oris)	Yes	No
Prevotella ruminicola	Yes	No

Prevotella tannerae	Yes	No
Propionibacterium acnes	Yes	Yes
Propionibacterium avidium	Yes	No
Propionibacterium granulosum	Yes	No
Propionibacterium lymphophilum	Yes	No
Propionibacterium propionicum (Arachnia propionica)	Yes	No
Proteus mirabilis	No	No
Proteus morganii	No	No
Proteus penneri	No	No
Proteus rettgeri	No	No
Proteus vulgaris	No	No
Providencia alcalifaciens	No	No
Providencia rettgeri	No	No
Providencia rustigianii	No	No
Providencia stuartii	No	No
Pseudoalteromonas piscicida (Pseudomonas piscicida)	Yes	No
Pseudomonas aeruginosa	Yes	Yes
Pseudomonas alcaligenes	Yes	No
Pseudomonas anguilliseptica	Yes	Yes
Pseudomonas mendocina	Yes	No
Renibacterium salmoninarum	No	No
Rhodococcus equi	Yes	No
Rickettsia africa	Yes	No
Rickettsia akari	Yes	No
Rickettsia australis	Yes	No
Rickettsia belli	Yes	No
Rickettsia canada	Yes	No
Rickettsia conorii	Yes	Yes
Rickettsia helvetica	Yes	No
Rickettsia japonica	Yes	No
Rickettsia massiliae	Yes	No
Rickettsia montana	Yes	No
Rickettsia parkeri	Yes	No
Rickettsia prowazekii	Yes	No
Rickettsia rhipicephali	Yes	No
Rickettsia rickettsi	Yes	No
Rickettsia sibirica	Yes	Yes
Rickettsia typhi	Yes	No
Salmonella choleraesuis	No	No
Sanguibacter inulinus	Yes	Yes
Sanguibacter keddieii	Yes	No
Sanguibacter suarezzii	Yes	No

Selenomonas artemidis	Yes	No
Selenomonas dianae	Yes	No
Selenomonas flueggei	Yes	No
Selenomonas infelix	Yes	No
Selenomonas noxia	Yes	No
Serpulina hyodysenteriae	No	No
Serpulina pilosicoli	No	No
Serratia grimesii	Yes	No
Serratia marcescens	Yes	No
Serratia proteamaculans	Yes	No
Serratia rubidaea	Yes	No
Shewanella alga	Yes	No
Shigella boydii	Yes	No
Shigella dysenteriae	Yes	No
Shigella flexneri	Yes	No
Shigella sonnei	Yes	No
Sphingobacterium multivorum (Flavobacterium multivorum)	Yes	No
Sphingobacterium spiritivorum (Flavobacterium spiritivorum)	Yes	No
Sphingobacterium thalpophilum (Flavobacterium thalpophilum)	Yes	No
Sphingomonas parapaucimobilis	Yes	No
Sphingomonas paucimobilis	Yes	No
Spiroplasma mirum	No	No
Staphylococcus aureus	Yes	Yes
Staphylococcus epidermidis	No	No
Staphylococcus felis	No	No
Staphylococcus haemolyticus	No	No
Staphylococcus hyicus	No	No
Staphylococcus intermedius	No	No
Staphylococcus lugdunensis	No	No
Staphylococcus pasteuri	No	No
Staphylococcus saprophyticus	No	No
Staphylococcus scheiferi	No	No
Stenotrophomonas maltophilia	Yes	Yes
Streptobacillus moniliformis	No	No
Steptococcus acidominimus	Yes	No
Steptococcus agalactiae	Yes	No
Steptococcus bovis	Yes	No
Steptococcus canis	Yes	No
Steptococcus constellatus	Yes	No
Steptococcus difficile	Yes	No
Steptococcus dysgalactiae	Yes	No
Steptococcus equi	Yes	No

Steptococcus gallolyticus	Yes	Yes
Steptococcus iniae	Yes	No
Steptococcus intermedius	Yes	No
Steptococcus mutans	Yes	No
Steptococcus oralis	Yes	No
Steptococcus parasanguis	Yes	No
Steptococcus phocae	Yes	No
Steptococcus pneumoniae	Yes	No
Steptococcus porcinus	Yes	No
Steptococcus pyogenes	Yes	No
Steptococcus salivarius	Yes	Yes
Steptococcus sanguis	Yes	No
Steptococcus sobrinus	Yes	No
Steptococcus suis	Yes	No
Streptomyces somaliensis	Yes	No
Tatlockia maceachernii (Legionella maceachernii)	No	No
Tatlockia micdadei (Legionella micdadei)	No	No
Tatumella ptyseos	No	No
Taylorella equigenitalis (Haemophilus equigenitalis)	No	No
Tissierella praeacuta (Bacteroides praeacutus)	No	No
Treponema carateum	Yes	No
Treponema denticola	Yes	No
Treponema maltophilum	Yes	No
Treponema pallidum	Yes	No
Treponema paraluiscuniculi	Yes	No
Treponema pectinovorum	Yes	No
Treponema pertenue	Yes	No
Treponema socranskii	Yes	No
Treponema vincentii	Yes	No
Tsukamurella inchonensis	No	No
Tsukamurella pulmonis	No	No
Turicella otitidis	No	No
Ureaplasma diversum	No	No
Ureaplasma urealyticum	No	No
Vagococcus salmoninarum	No	No
Vagococcus parvula	No	No
Vibrio alginolyticus (Beneckea alginolytica)	Yes	No
Vibrio carchariae	Yes	No
Vibrio choleraesuis	Yes	No
Vibrio cincinnatiensis	Yes	No

Vibrio fluvialis	Yes	No
Vibrio furnissii	Yes	No
Vibrio holisae	Yes	No
Vibrio ichthyoenteri	Yes	No
Vibrio metschnikovii	Yes	No
Vibrio mimicus	Yes	No
Vibrio ordalii	Yes	No
Vibrio parahaemolyticus (Beneckea parahaemolytica)	Yes	No
Vibrio salmonicida	Yes	No
Vibrio trachuri	Yes	No
Vibrio vulnificus (Beneckea vulnifica)	Yes	No
Yersinia enterocolitica	No	No
Yersinia frederiksenii	No	No
Yersinia intermedia	No	No
Yersinia kristensenii	No	No
Yersinia pestis	No	No
Yersinia pseudotuberculosis	No	No
Yersinia ruckeri	No	No

Appendix VI

Table A8.Full list of harmful algae screened for in the eukaryotic biofilm and planktonic communities

Harmful algae	Genus level	Species level
Alexandrium andersoni	Yes	No
Alexandrium australiense	Yes	No
Alexandrium balechii	Yes	No
Alexandrium catenella	Yes	No
Alexandrium excavatum	Yes	No
Alexandrium fundyense	Yes	No
Alexandrium hiranoi	Yes	No
Alexandrium ibericum	Yes	No
Alexandrium lustitanicum	Yes	No
Alexandrium minutum	Yes	No
Alexandrium monilatum	Yes	No
Alexandrium ostenfeldii	Yes	No
Alexandrium pacificum	Yes	No
Alexandrium peruvianum	Yes	No
Alexandrium pseudogonyaulax	Yes	No
Alexandrium tamarense	Yes	No
Alexandrium tamiyavanichii	Yes	No
Alexandrium taylori	Yes	No
Alexandrium Halim	Yes	No
Amphidinium carterae	No	No
Amphidinium gibbosum	Yes	No
Amphidinium klebsii	Yes	No
Amphidinium operculatum	Yes	No
Amphidoma languida	No	No
Amphioauropus	No	No
Amphora coffeaeformis	Yes	Yes
Anabaena bergii	No	No
Anabaena macrospora	No	No
Anabaenopsis milleri	No	No
Aphanizomenon flosaquae	No	No
Arthrospira fusiformis	No	No
Aureococcus anophagefferens	Yes	Yes
Aureoumbra lagunensis	No	No
Azadinium poporum	No	No
Azadinium spinosum	No	No
Chattonella globosa	No	No
Chattonella japonica	No	No
Chattonella marina	No	No

Chattonella subsalsa	No	No
Chattonella verruculosa	No	No
Chrysochromulina leadbeateri	Yes	No
Chrysochromulina polylepis	Yes	No
Chrysosporum bergii	No	No
Cochlodinium heterolobatum	No	No
Cochlodinium polykrikoides	No	No
Coelosphaerium kuetzingianum	No	No
Coolia tropicalis	No	No
Cryptomonas lima	No	No
Cyanobium bacillare	No	No
Cylindrospermopsis raciborskii	No	No
Cylindrospermum stagnale	No	No
Dinophysis acuminata	No	No
Dinophysis acuta	No	No
Dinophysis boehmii	No	No
Dinophysis borealis	No	No
Dinophysis caudata	No	No
Dinophysis fortii	No	No
Dinophysis infundibulum	No	No
Dinophysis miles	No	No
Dinophysis mitra	No	No
Dinophysis norvegica	No	No
Dinophysis ovum	No	No
Dinophysis reniformis	No	No
Dinophysis rotundata	No	No
Dinophysis saccula	No	No
Dinophysis tripos	No	No
Dinophysis ventrecta	No	No
Dolichospermum circinale	No	No
Dolichospermum flosaquae	No	No
Dolichospermum lemmermannii	No	No
Dolichospermum macrosporum	No	No
Dolichospermum mendotae	No	No
Dolichospermum planctonicum	No	No
Dolichospermum sigmoideum	No	No
Dolichospermum spiroides	No	No
Entomosigma akashiwo	No	No
Exuviaella cassubica	Yes	No
Exuviaella hoffmanianum	Yes	No
Exuviaella lima	Yes	No
Exuviaella maculosum	Yes	No
Exuviaella marina	Yes	No
Exuviaella minima	Yes	No
Fibrocapsa japonica	Yes	No
Frustulia coffeaeformis	Yes	No

Fukuyoa yasumotoi	No	No
Gambierdiscus australes	No	No
Gambierdiscus balechii	No	No
Gambierdiscus belzeanus	No	No
Gambierdiscus caribaeus	No	No
Gambierdiscus carolinianus	No	No
Gambierdiscus carpenteri	No	No
Gambierdiscus cheloniae	No	No
Gambierdiscus excentricus	No	No
Gambierdiscus lapillus	No	No
Gambierdiscus pacificus	No	No
Gambierdiscus polynesiensis	No	No
Gambierdiscus ruetzleri	No	No
Gambierdiscus scabrosus	No	No
Gambierdiscus silvae	No	No
Gambierdiscus toxicus	No	No
Geitlerinema amphibium	No	No
Geitlerinema lemmermannii	No	No
Gessnerium balechii	No	No
Gessnerium catenella	No	No
Gessnerium catenellum	No	No
Gessnerium mochimaensis	No	No
Gessnerium ostenfeldii	No	No
Gessnerium tamarensis	No	No
Gloeotrichia echinulata	No	No
Goniodoma ostenfeldii	No	No
Goniodoma pseudogonyaulax	No	No
Gonyaulax balechii	Yes	No
Gonyaulax catenella	Yes	No
Gonyaulax excavata	Yes	No
Gonyaulax grindleyi	Yes	No
Gonyaulax monilata	Yes	No
Gonyaulax ostenfeldii	Yes	No
Gonyaulax schilleri	Yes	No
Gonyaulax spinifera	Yes	Yes
Gonyaulax tamarensis	Yes	No
Gonyaulax taylorii	Yes	No
Gymnodinium breve	Yes	No
Gymnodinium brevisulcatum	Yes	No
Gymnodinium brevs	Yes	No
Gymnodinium catenatum	Yes	No
Gymnodinium cladochromum	Yes	No
Gymnodinium galatheanum	Yes	No
Gymnodinium micum	Yes	No
Gymnodinium mikimotoi	Yes	No
Gymnodinium nagasakiense	Yes	No

Gymnodinium veneficum	Yes	No
Gyrodinium corsicum	Yes	No
Gyrodinium galatheanum	Yes	No
Halamphora coffeaeformis	Yes	No
Hemieutreptia antiqua	No	No
Heterocapsa circularisquama	No	No
Heterosigma akashiwo	No	No
Heterosigma carterae	No	No
Hornellia marina	No	No
Kamptonema formosum	No	No
Karenia bicuneiformis	Yes	No
Karenia bidigitata	Yes	No
Karenia brevisulcatum	Yes	No
Karenia concordia	Yes	No
Karenia cristata	Yes	No
Karenia digitata	Yes	No
Karenia mikimotoi	Yes	Yes
Karenia papilonacea	Yes	No
Karenia selliformis	Yes	No
Karenia umbella	Yes	No
Karlodinium armiger	No	No
Limnothrix redekei	No	No
Lingulodinium polyedra	No	No
Luciella masanensis	No	No
Lyngbya majuscula	No	No
Margalefidinium polykrikoides	No	No
Microcystis aeruginosa	No	No
Microcystis botrys	No	No
Microcystis flosaquae	No	No
Microcystis ichthyoblabe	No	No
Microcystis panniformis	No	No
Microcystis viridis	No	No
Microcystis wesenbergii	No	No
Nitzschia actydrophila	Yes	Yes
Nitzschia bizertensis	Yes	No
Nitzschia delicatissima	Yes	No
Nitzschia fraudulenta	Yes	No
Nitzschia multistriata	Yes	No
Nitzschia pungens	Yes	No
Nitzschia seriata	Yes	No
Nitzschia turgidula	Yes	No
Nodularia spumigena	Yes	No
Olisthodiscus carterae	No	No
Ostreopsis fattorussoi	No	No
Ostreopsis heptagona	No	No
Ostreopsis lenticularis	No	No

Ostreopsis mascarensis	No	No
Ostreopsis ovata	No	No
Ostreopsis rhodesiae	No	No
Ostreopsis siamensis	No	No
Peridinium reticulatum	Yes	No
Pfiesteria piscicida	Yes	Yes
Pfiesteria shumwayae	Yes	No
Phaeocystis globosa	No	No
Phaeocystis pouchetii	No	No
Phalacroma mitra	No	No
Pheopolykrikos hartmannii	No	No
Phormidium formosum	No	No
Phormidium uncinatum	No	No
Planktothrix rubescens	No	No
Polykrikos hartmannii	Yes	No
Prorocentrum arabianum	No	No
Prorocentrum arenarium	No	No
Prorocentrum belizeanum	No	No
Prorocentrum borbonicum	No	No
Prorocentrum caipirignum	No	No
Prorocentrum cassubicum	No	No
Prorocentrum concavum	No	No
Prorocentrum cordatum	No	No
Prorocentrum emarginatum	No	No
Prorocentrum faustiae	No	No
Prorocentrum hoffmanianum	No	No
Prorocentrum lima	No	No
Prorocentrum maculosum	No	No
Prorocentrum marinum	No	No
Prorocentrum mexicanum	No	No
Prorocentrum rhathymum	No	No
Prorocentrum texanum	No	No
Protoceratium reticulatum	No	No
Protogonyaulax catenella	No	No
Prymnesium calathiferum	No	No
Prymnesium faveolatum	No	No
Prymnesium parvum	No	No
Prymnesium patellifera	No	No
Prymnesium polylepis	No	No
Prymnesium zebrinum	No	No
Pseudochattonella farcimen	No	No
Pseudochattonella verruculosa	No	No
Pseudo-nitzchia abrensis	Yes	No
Pseudo-nitzchia australis	Yes	Yes
Pseudo-nitzchia batesiana	Yes	No
Pseudo-nitzchia brasiliana	Yes	No

Pseudo-nitzchia caciantha	Yes	No
Pseudo-nitzchia calliantha	Yes	No
Pseudo-nitzchia cuspidata	Yes	No
Pseudo-nitzchia delicatissima	Yes	No
Pseudo-nitzchia fraudulenta	Yes	No
Pseudo-nitzchia fukuyoi	Yes	No
Pseudo-nitzchia galaxiae	Yes	No
Pseudo-nitzchia granii	Yes	No
Pseudo-nitzchia hasleana	Yes	No
Pseudo-nitzchia kodamae	Yes	No
Pseudo-nitzchia lundholmiae	Yes	No
Pseudo-nitzchia multiseries	Yes	No
Pseudo-nitzchia multistriata	Yes	No
Pseudo-nitzchia obtusa	Yes	No
Pseudo-nitzchia plurisecta	Yes	No
Pseudo-nitzchia pseudodelicatissima	Yes	No
Pseudo-nitzchia pungens	Yes	Yes
Pseudo-nitzchia seriata	Yes	No
Pseudo-nitzchia simulans	Yes	No
Pseudo-nitzchia subfraudulenta	Yes	No
Pseudo-nitzchia subpacifica	Yes	No
Pseudo-nitzchia turgidula	Yes	Yes
Pseudopfiesteria shumwayae	No	No
Ptychodiscus brevis	No	No
Pyrodinium bahamense	No	No
Pyrodinium bahamense	No	No
Pyrodinium balechii	No	No
Pyrodinium minutum	No	No
Pyrodinium monilatum	No	No
Pyrodinium phoneus	No	No
Pyrodinium schilleri	No	No
Raphidiopsis curvata	No	No
Raphidiopsis mediterranea	No	No
Snowella lacustris	No	No
Takayama cladochroma	No	No
Tetraspora poucheti	No	No
Triadinum ostenfeldii	No	No
Trichormus variabilis	No	No
Verrucophora farcimen	No	No
Vicicitus globosus	No	No
Vulcanodinium rugosum	No	No
Woloszynskia micra	Yes	No