Marine Antifouling Coatings Determine Biofilm Community Composition and Microbiome Development in Settling Invertebrates

Submitted by

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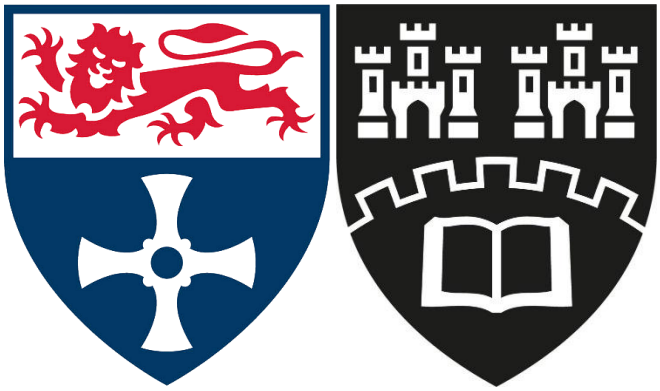

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Abstract

This study investigated the impact of biocidal and non-biocidal marine anti-fouling coatings on the microbial composition and functionality of associated biofilms, marine invertebrates and their larvae using 16S rRNA gene sequencing. This is complemented by exploratory shotgun sequencing which was used to identify resistance genes present within the anti-fouling treatments.

Differences between biocidal and non-biocidal biofilm treatments were identified, with unique ASVs found in each treatment. Although there was a high level of community overlap between treatments, there were diversity distinctions between treatments. Predicted functional analysis also demonstrated distinct differences in their antimicrobial resistance potential between biocidal and non-biocidal treatments. Shotgun sequencing analysis identified polymyxin and multi-drug as the dominant resistance gene types.

In the barnacle *Semibalanus balanoides*, the adult microbiome acquisition upon settlement was heavily influenced by the settling substrate, forming beta-diversity-rated distinct communities, where the biggest distinction in the microbiome was between the planktonic cyprid and calcification.

In established adult-stage *Ciona intestinalis* and *Bugula neritina*, the coating they were attached to impacted their associated microbial communities. *C. intestinalis* on biocidal coatings with booster biocides demonstrated a significant reduction in dominant taxa compared to the other treatments. Similarly, in *B. neritina* the dominant taxa composition was significantly different between those with booster biocides and the other treatments. The microbiome AMR potential was characterised using shotgun sequencing and lab culture. Ampicillin and ciprofloxacin resistance were reduced in biocidal coatings in bryozoan samples, whereas kanamycin resistance was more prevalent in biocidal treatments. Larval microbiomes were distinct from their parents; however, they demonstrated both trans-generational transfer and uptake from the surrounding environment.

The findings from this study not only contribute to the understanding of ecological consequences of antifouling strategies, but also offer insights into the potential implications of marine microbiome change.

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Table of Abbreviations

ABBREVIATION	DEFINITION
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
MRG	Metal(loid) Resistance Gene
MGE	Mobile Genetic Element
VC	VC Offshore anti-fouling paint
TRI	Trilux33 anti-fouling paint
SIL	Silic One anti-fouling paint
COP	Coppercoat anti-fouling paint
PRI	Primer paint
ECO	Ecopower anti-fouling paint
ANOSIM	Analysis Of Similarities
BLAST	Basic Local Alignment Search Tool
DNA	Desoxyribonucleic Acid
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
WHO	World Health Organisation
ASV	Amplicon Sequence Variants
PCOA	Principal Coordinates Analysis
rRNA	Ribosomal RNA
EUCAST	European Committee on Antimicrobial Susceptibility Testing

Preface

The current project was a collaboration between Newcastle University, Northumbria University, Essex University, and CASE partner PML applications. It was funded by the NERC OnePlanet DTP and supplemented by PML Applications, and a grant supplied by Newcastle University as part of the doctoral enhancement fund.

Newcastle University provided the base of the research for this thesis, providing financial and supervisory support, as well as office and laboratory space. NUOMICS at Northumbria University provided sequencing expertise and services for all sequencing data produced in this thesis, as well as financial assistance with sequencing costs. PML Applications provided financial support outside and during placements, as well as office and lab facilities, lab training, guidance and support, field deployment and maintenance of settlement panels. Essex University provided supervisory guidance and an extra field site. Bioinformatic training was undertaken with NEOF and Cloud span courses, as well as guidance and advice from NUOMICs.

The main objective of the study was to determine if biocidal anti-fouling coatings alters the microbiome compositions and functions associated with marine fouling invertebrates and assess if this increases the presence of resistance genes and pathogens. An assessment was also done to determine the risk of transfer of unfavourable genes/taxa trans-generationally in marine invertebrates.

Project aims and objectives

Each chapter covers a portion of the above objective and were set out as follows:

Chapter 1: Marine fouling communities and their potential as AMR reservoirs and pathogens

A review of anti-fouling technologies' effects on marine microbiomes was conducted to gain an understanding of how biocides may influence marine microbiomes and the potential underlying mechanisms.

Chapter 2: Characterising The Colonising Biofilm Communities on Different Anti-fouling Coatings.

Wild biofilms were grown on settlement panels at the port of Blyth on biocidal and non-biocidal anti-fouling coatings. The colonising biofilm biomes were characterised based on community composition.

Aims:

- Determine how the colonising biofilm changes on different anti-fouling coatings.
- Determine if antimicrobial resistance genes are more prevalent on biocidal vs non-biocidal coatings.

Objectives:

- Perform 16S analyses to review and compare community composition between coatings and over time.
- Assess diversity between treatments.
- Identify pathogenic or virulent species.
- Use exploratory shotgun sequencing to investigate what antimicrobial resistance genes are present and if there are differences in resistance profiles between treatments.
- Provide resistome risk scores and determine risk differences between the biocidal and non-biocidal treatments.

Chapter 3: Anti-fouling coatings influence microbiome acquisition of settling *Semibalanus balanoides.*

Different developmental stages of *Semibalanus balanoides* were collected during the settlement season of 2022 from different coatings in the port of Blyth.

Aims:

- To determine if biocidal coatings influence the microbiome acquisition of settling *Semibalanus balanoides* cyprids.

Objectives:

- Collect settling cyprids from planktonic cyprids through to juvenile barnacles on different anti-fouling coatings.
- Use 16S data to conduct community comparisons between developmental stages, treatments, surrounding seawater and biofilm.
- Identify the differences in community composition between biocidal and non-biocidal treatments.

Chapter 4: Fouling invertebrate-associated microbial communities change with host exposure to biocidal coatings.

Fouling invertebrates were allowed to settle on settlement panels coated with anti-fouling coatings in Millbay Marina, Plymouth. Microbiomes from established fouling communities were investigated. Antibiotic resistance assays of whole microbiomes were also conducted to provide an assessment of resistance profiles.

Aims:

- Determine how the microbiomes of fouling invertebrates are influenced by biocidal and non-biocidal anti-fouling coatings.
- Determine if antimicrobial resistance genes are more prevalent on biocidal vs non-biocidal coatings.
- Assess antibiotic resistance profiles between invertebrate microbiomes grown on biocidal or nonbiocidal coatings.

Objectives:

- Sample from multiple fouling species from different anti-fouling, biocidal and non-biocidal coatings.
- Identify and compare microbiome compositional changes within the microbiomes of fouling invertebrates from biocidal and nonbiocidal coatings.
- Identify antibiotic resistance profiles and any differences between biocidal and non-biocidal grown invertebrate microbiomes.

Chapter 5: Microbiome transfer from fouling invertebrate adults to larvae.

Fouling marine invertebrates were collected from the wild from Millbay marine, Plymouth, as well as lab-reared barnacles, and were induced in the laboratory to spawn.

Aims:

- To assess the proportional rate of transfer of the microbiome from parents to offspring in multiple fouling species.

Objectives:

- To induce spawning of brooding marine invertebrates in controlled conditions in the laboratory, and in-vitro fertilisation of gamete spawning *C. intestinalis.*
- Compare community composition of the parental and offspring microbiome.
- Quantify the rate of trans-generational transfer using SourceTracker.

Chapter 6: Final Discussion

Final discussion and overview of the research conducted in this thesis. Outline of possible implications of the research results and proposal of future research directions to follow.

Chapter 1: Marine Fouling Communities and Their Potential as AMR Reservoirs and Pathogens

1.1 Biofouling Background

Marine Biofouling is the unwanted colonization of underwater structures such as pipes or ship hulls, by micro-organisms (e.g. viruses, bacteria, cyanobacteria, fungi, protozoa and microalgae) and/or macro-organisms (e.g. hard fouling species such barnacles, mussels and tubeworms and soft-fouling organisms such as non-calcareous algae, sponges, anemones, tunicates and hydroids) (Wahl, 1989). Marine biofouling will happen to varying degrees on any available submerged surface, from biofilms to full mixed micro and macro communities.

Marine biofouling has typically been characterised into five successional phases: (1) adsorption of dissolved organic and inorganic molecules, (2) colonization by prokaryotes and unicellular eukaryotes (e.g. diatoms, flagellates, and ciliates), (3) biofilm formation and EPS production (4) complex community formation including multicellular species, microalgae, debris and sediment, and (5) recruitment of invertebrate larvae and algal spores (Figure 1.1). While this description is classed as a “successional model” of fouling, in reality, it is widely accepted that this process is more dynamic and can happen in succession, overlap, or occur in parallel (Connell and Slatyer, 1977; Cooksey and Wigglesworth-Cooksey, 1995; Dobretsov, 2010). The “probabilistic” model of fouling identifies that colonisation occurs even in the absence of a “stage” and does not impede the occurrence of another (Clare *et al.*, 1992; Maki and Mitchell, 2003; Vinagre *et al.*, 2020) (Figure 1.2). A prime example of this is seen in Roberts *et al.*, (1991) which demonstrated the settlement of macro-foulers such as the acorn barnacle and bryozoan species were still able to settle on a substrate without the presence of a conditioning biofilm.

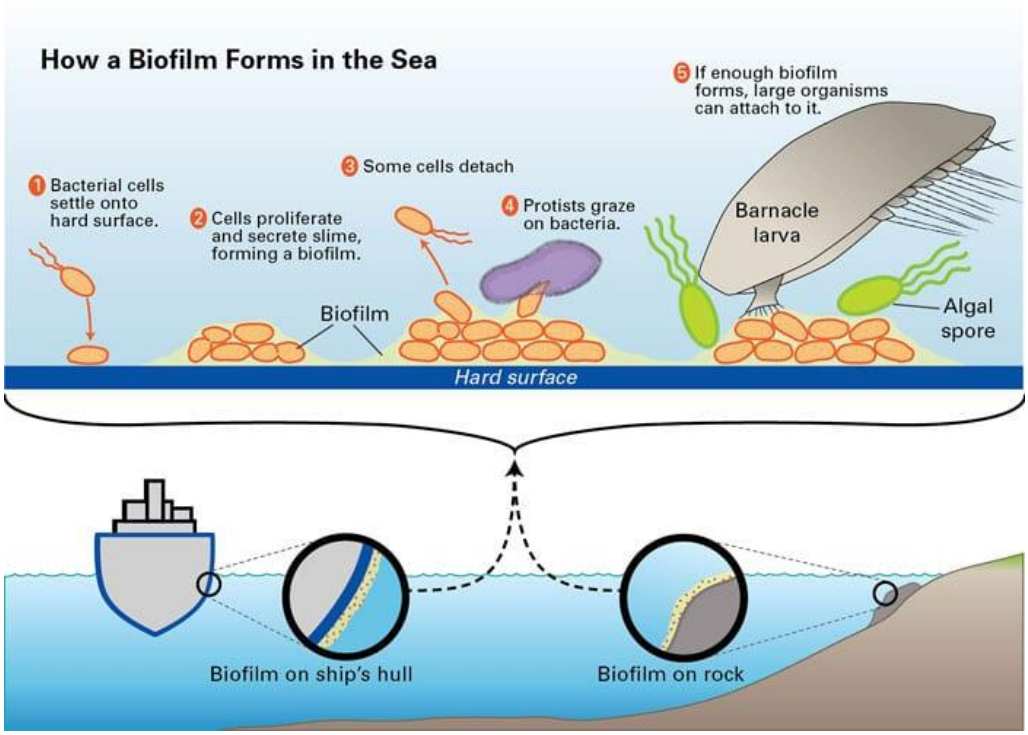


Figure 1.1. Illustration of successional biofilm formation in the marine environment. Illustration by Jack Cook, Wood Hole Oceanographic Institution

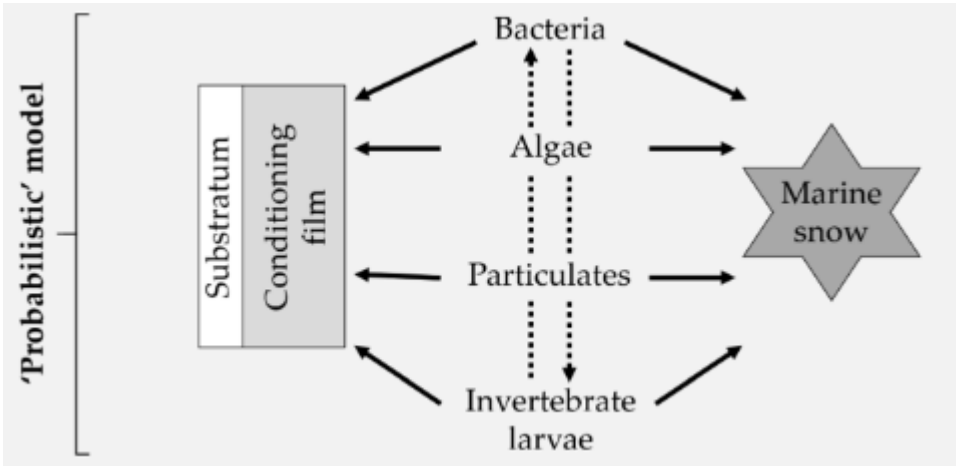


Figure 1.2 Schematic of the Probabilistic model of biofouling from Vinagre et al., (2020)

Although macro-foulers can settle without a preconditioned biofilm, there is also significant evidence that bacterial biofilms can enhance and promote macrofouling through the production of settlement cues (Rodriguez *et al.*, 1995; Wieczorek, Clare and Todd, 1995; Hadfield, 2011).

The composition of fouling communities are influenced by a mixture of biotic and abiotic factors and will vary across geography and season. In general, the rate of biofouling growth increases with temperature, usually following seasonal trends and reproductive cycles (Vinagre *et al.*, 2020). Polar regions will generally have low rates of biofouling, compared to tropical locations, where the warm temperatures allow for year-round reproduction of many marine invertebrates (Hellio and Yebra, 2009).

Fouling causes a breadth of problems, often at the cost of economic losses as well as negative environmental impacts, thus posing a worldwide problem (Callow, 1990; ICES, 2019). The use of anti-fouling paints has been the primary method for hundreds of years to combat unwanted fouling. Shipping, which accounts for approximately 90% of world trade (ICS and ICF, 2009), is predicted to face costs of up to $60 billion a year for biofouling-associated increased fuel consumption due to structural damage and increased drag. Without effective coatings, emissions of CO_2 would increase by 284 million tons per year (Bressy and Lejars, 2014), equivalent to an economy the size of Germany (3% of the global total) (Smith *et al.*, 2014). Considering the scale of the maritime industry, the significant economic costs of hull fouling has been the primary driving force behind the development of anti-fouling technologies, constituting a global industry that is worth approximately US$ 4 billion annually (Dafforn, Lewis and Johnston, 2011). In aquaculture, another multi-billion dollar industry, spends 5–10% of production costs mitigating biofouling, or up to US$3 billion per year (Fitridge *et al.*, 2012). Impacts on aquaculture not only include damage to infrastructure but can lead to sub-optimal conditions leading to a reduction of quality and quantity of cultured stock, as well as the spread of disease being harboured within fouling species (Drake *et al.*, 2005; Kroeck and Montes, 2005; Abollo *et al.*, 2008; Neill, Heesch and Nelson, 2008; Fitridge *et al.*, 2012; Molloy *et al.*, 2013; Bannister *et al.*, 2019). Due to the nature of problem-fouling species often being on transportable substrates (ship hulls), they are often transported to areas beyond their natural dispersal and occurrence. This poses a biosecurity risk, enabling the spread of invasive species, and associated pathogens (Hewitt, Gollasch and Minchin, 2009; Bell *et al.*, 2011; Davidson *et al.*, 2014; ICES, 2019; Georgiades *et al.*, 2020, 2021).

1.1.1 Invasive Species

One of the biggest environmental threats from biofouling is the transport of alien species to areas beyond their natural dispersal, which then go on to survive and

establish new populations. These new reproductively viable populations can become invasive, usually out-competing native species, and often cause irreversible ecological and economic damage. Aquatic invasive species are transported to new bioregions by ships mainly through hull fouling or ballast water, often carrying pathogens with them (Brockerhoff and McLay, 2008; Hewitt, Gollasch and Minchin, 2009; Georgiades *et al.*, 2021). For example, the tunicate *Botrylloides violaceus* is a colonial tunicate native to the north-west Pacific, however, due to biofouling transport, has established itself as an invasive species in several temperate regions around the globe (Simkanin *et al.*, 2012, 2013). Similarly, *Didemnum vexillum*, originally native to Japan, has steadily increased its range to include New Zealand, North America and Europe. Both these tunicate species have had negative impacts on commercial aquaculture practices through heavy growth, often obstructing valve openings or siphons of culturable stock (Ferguson *et al.*, 2017). Not only are these species pests in their own being, but they can harbour pathogens such as osterid herpesvirus, *Vibrio* spp, *Bonamia ostreae* and *Minchinia* spp, that affect commercial bivalves (*Ostrea edulis, Crassostrea gigas, Cerastoderma edule*) (Lane, Jones and Poulin, 2018; Costello *et al.*, 2021; Georgiades *et al.*, 2021).

The spread of invasives and associated pathogens, however, isn't limited to hull fouling and ballast water. They can be transported within the aquaculture industry, as stock, on stock, and harbouring within. For example, the haplosporidian *Bonamia exitiosa* has been reported in European flat oysters on the Galician coast. Phylogenetic analyses determined it belongs to a southern hemisphere clade which has been brought over as a result of repeat shipment of oysters from southern waters of the USA (Abollo *et al.*, 2008). A more modern vector of spread, is the fouling species on plastic debris and microplastics. Viršek *et al.*, (2017) identified pathogenic fish bacteria *Aeromonas salmonicida* on microplastics, being transported long distances along ocean currents.

1.1.1.1 Regulation: Invasive Species

A worldwide problem requires international scale co-operation. Collaboration is required from various bodies of power, including governments, non-governmental organisations, economic sectors, and international treaty organisations. The duties of states have been outlined in a global framework by the UN Convention on the Law of the Sea (UNCLOS), which specifically states "States shall take all measures necessary

to prevent, reduce and control pollution of the marine environment resulting from the use of technologies under their jurisdiction or control, or the intentional or accidental introduction of species, alien or new, to a particular part of the marine environment, which may cause significant and harmful changes thereto" (STARACE, 2012).

As a result, the International Marine Organisation (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments, 2004 (BWM Convention) as a commitment to minimise the transport of invasive species (specifically through shipping and ballast water). In 2012, guidelines on how to minimise biofouling were published by the IMO and the Marine Environment Protection Committee (MEPC). The key point on how biofouling can be minimised is the use of an appropriate anti-fouling coating system, as well as good maintenance (MEPC, 2012, 2023). However, standards and legal requirements for anti-fouling systems can vary internationally.

1.1.2 Use of Anti-fouling Systems and Biocides

Anti-fouling paint has traditionally used biocides to prevent colonization through toxicity and fits within two categories: metallic and non-metallic compounds. Biocides are defined by the biocides directive (98/8/EC) (European Parliament and of the Council., 1998) as active substances or preparations that are intended to destroy, deter, render harmless and exercise control or prevent the action of any other harmful organism through chemical or biological means.

Tributyltin (TBT) dominated as the active ingredient in anti-fouling paints from the 1960s to 1990s. Although TBT is a very efficient biocide, it has been well documented to cause adverse environmental effects, including abnormal shell growth in commercially important oysters (Scammell, Batley and Brockbank, 1991; Stephenson, 1991), to impo-sex in dogwhelks (Gibbs, Bryan and Pascoe, 1991; Schøyen *et al.*, 2019; Bryan, Gibbs and Burt, 2021). Consequently, the use of TBT and other organotin biocides has been banned under the IMO's convention on the control of harmful anti-fouling systems on ships (AFS, 2001). However it wasn't until 2003 that a ban on further application on ships came into force, then in 2008 where the active presence of TBT was finally banned (from ships that had pre-existing TBT paint applied) (Thomas, 2009). Without the use of TBT, the anti-fouling market was propelled to

favour alternative biocides (see Table 1.1). However, some alternatives have been found to be just as damaging and have been banned in various countries around the world. For example, Diuron and Irgarol 1051 dominated as the active ingredient in anti-fouling paints in the UK (50% and 30% respectively) in the 1990s (Environment Agency, 1998), however due to their negative environmental impacts (Chesworth, Donkin and Brown, 2004), in 2001, legislative measures restricted their use, limiting anti-fouling agents on small (<25m) vessels to dichlofluanid, zinc pyrithione and zineb at the time (Cresswell *et al.*, 2006).

Table 1.1 Status of common biocidal components in anti-fouling paints as of august 2024 (adapted from HSE database: https://www.hse.gov.uk/biocides/copr/approved.htm). Rows coloured in blue indicate substances under review.

Substance name	CAS number	Approval start date	Approval expiry date	Renewal submission deadline	Approval/ Assessment status
Copper flakes (coated with aliphatic acids)	7440-50-8	01/01/18	31/12/25	Renewal not supported	Approved
Dichloro-N-[(dimethylamino)sulphonyl] fluoro-N-(ptolyl)methanesulphenamide (Tolylfluanid)	731-27-1	01/07/16	31/12/25	Renewal not supported	Approved
N-(Dichlorofluoromethylthio)-N',N'-dimethyl-N-phenylsulfamide (Dichlofluanid)	1085-98-9	01/11/18	31/12/25	Renewal not supported	Approved
Zinc ethylenebis(dithiocarbamate) (polymeric) (Zineb)	12122-67-7	01/01/16	31/12/25	Renewal not supported	Approved
4,5-Dichloro-2-octylisothiazol-3(2H)-one (4,5-Dichloro-2-octyl-2H-isothiazol-3-one (DCOIT))	64359-81-5	01/01/16	31/01/27		Approved - under renewal
4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile (Tralopyril)	122454-29-9	01/04/15	31/01/27		Approved - under renewal
Bis(1-hydroxy-1H-pyridine-2-thionato-O,S)copper (Copper pyrithione)	14915-37-8	01/10/16	31/01/27		Approved - under renewal

Copper thiocyanate	1111-67-7	01/01/18	31/01/27		Approved - under renewal
Dicopper oxide	1317-39-1	01/01/18	31/01/27		Approved - under renewal
Medetomidine	86347-14-0	01/01/16	31/01/27		Approved - under renewal
Copper, powder	7440-50-8				Under review
Pyrithione zinc (Zinc pyrithione)	13463-41-7				Under review
Free radicals generated in situ from ambient air or water					Under assessment

The 2001 AFS convention not only eliminates traditionally used organotin compounds, but calls for ongoing assessment of other future harmful biocides, some of which have seen the market and already faced restrictions by 2008. Since the AFS convention, regulation hasn't really changed, but in July 2017, at the IMO 71st annual meeting, the MEPC approved a new output to amend Annex 1 to the AFS Convention to include controls on Cybutryne (aka Irgarol 1051). Then in 2021, further controls were added again for Cybutryne that came into force in 2023, which effectively bans its use or order to be removed at a ship's next anti-fouling renewal date (MEPC, 2023). Currently, biocide alternatives for anti-fouling paints are typically based on copper compounds (e.g. cuprous oxide and copper thiocyanate), supplemented with booster biocides to control copper-resistant fouling organisms (Guardiola *et al.*, 2012; Amara *et al.*, 2018). As of August 2024, around 13 substances are approved for use as biocidal additives in professional and amateur anti-fouling products by the HSE in the UK (Table 1.1). However, two of these are under review and one under assessment. Zinc pyrithione was recently declared non-approved for type 2 uses (disinfectants and algaecides not intended for direct application to humans or animals), however still appears to be under review for type 21 uses (anti-fouling products).

1.1.3 Impacts of Anti-fouling Biocides in the Marine Environment

Anti-fouling paint is traditionally toxic by nature. As established, many anti-fouling biocides have been banned for their negative effects on non-target species and the surrounding environment. Depending on the biocide used, the biological effects will vary. A small selection of anti-fouling biocides dominate the market in many countries, and as a result, high concentrations have been found in areas of high yachting activity, particularly in marinas and sportive harbours (Konstantinou and Albanis, 2004).

Modern anti-fouling biocides are intended to be less harmful to the environment, however, their toxicity on several marine species remains unclear (Jacobson and Willingham, 2000; Braithwaite and Fletcher, 2005; Bellas, 2006; Mochida *et al.*, 2006; Cima, Bragadin and Ballarin, 2008), including their persistence within the environment (Thomas and Brooks, 2010; Chiavarini, Ubaldi and Cannarsa, 2014; Amara *et al.*, 2018).

The use of copper compounds has become the foundation of anti-fouling paint. It is toxic to most marine organisms, including molluscs, crustaceans, and algae. Copper can disrupt the development and growth of molluscs (e.g. *Crassostrea virginica* and *C. giga*) (Calabrese *et al.*, 1973; Al-Subiai *et al.*, 2011), behavioural changes in invertebrates such as *Scrobicularia plana* and *Hediste diversicolor* (Buffet *et al.*, 2011), and cause high mortality rates in crustaceans at different life stages (Alzieu *et al.*, 1980; Mochida *et al.*, 2006). In algae, copper can inhibit growth, block photosynthetic activity and cause visible lesions in macroalgae (Alzieu *et al.*, 1980; Dupraz *et al.*, 2018). The use of copper has survived changes in legislation due to its efficiency and its low tendency towards bioaccumulation due to its lipophilic properties (Voulvoulis, Scrimshaw and Lester, 1999). Even though there are concerns about copper concentrations on marine organisms, it is considered that the low bioavailability of copper ions released from anti-fouling paints still presents an acceptable profile that complies with environmental quality standards (Almeida, Diamantino and de Sousa, 2007).

The same cannot be said for some other anti-fouling biocides. Zinc pyrithione is one of the most common booster biocides today often used in conjunction with copper. Zinc pyrithione is allowed in most European waters, but is currently under review by the Biocidal Products Regulation (BPR) of the EU. It is also reported that zinc pyrithione is biodegraded or hyrdolysed into stable metabolites of their own toxicity. It also accumulates in the water column or sediment, if it hasn’t degraded under certain environmental conditions. Zinc pyrithione is highly toxic to some species of fish, echinoderms, crustacea, phyto- and zoo-plankton (Goka, 1999; Okamura *et al.*, 2002; Bellas, 2006; Jung *et al.*, 2017) and algal species, inhibiting growth, and effecting germination and growth responses (Koutsaftis and Aoyama, 2006; Myers *et al.*, 2006; Dupraz *et al.*, 2018; Soon *et al.*, 2019). Despite the short half-life of zinc pyrithione in the environment, a short pulse of exposure at a low concentration is sufficient to have

significant effects on a phytoplankton community (Soon *et al.*, 2019). Zinc pyrithione works by restricting ATP synthesis in prokaryotic cells and hindering membrane transport in bacteria (Chandler and Segel, 1978; Dinning *et al.*, 1998).

Biocides such as Sea-nine, Irgarol and Diruon are very toxic to a broad range of phyla at low concentrations (Scarlett *et al.*, 1997; Okamura *et al.*, 2000; Fernández-Alba *et al.*, 2002; Braithwaite and Fletcher, 2005; Amara *et al.*, 2018). Environmental accumulation of these biocides such as Irgarol have been linked with perturbations in coastal phytoplankton communities (Hall *et al.*, 2005). The presence of Diuron in the environment is not exclusive to anti-fouling however, and has been predominantly used as a herbicide in non-agricultural settings since the 1950s. Organotin compounds have been the most documented, and shown to have adverse effects on a breadth of invertebrate species (Amara *et al.*, 2018), from abnormal shell growth and failure of spat in oysters (Scammell, Batley and Brockbank, 1991; Stephenson, 1991; Alzieu, 2000b, 2000a), impotence of neogastropods and gastropods (Bryan, Gibbs and Burt, 2021), fertility and thus reduction of population in dogwhelks (Gibbs, Bryan and Pascoe, 1991) and reduced growth in mussels (Huang and Wang, 1995). Chlorothalonil is also particularly toxic to crustaceans at all life stages leading to eventual death (Bellas, 2006), as well as causing immunological dysfunction and reproductive complications in fish (Du Gas *et al.*, 2017).

1.1.4 Impacts of Anti-fouling Biocides on Microbial Communities

Increasing use of biocides in various industries, such as agriculture, aquaculture and shipping has raised concerns about their potential impact on microbial communities. Copper and zinc-based compounds have been demonstrated to have anti-microbial effects, inhibiting EPS production, biofilm formation, membrane damage (Tabrez Khan *et al.*, 2013), and change in community composition (Chen *et al.*, 2013; Ding *et al.*, 2019). However, the effect of these biocides can vary depending on their form and the type of microbial community it's affecting. For example, copper oxide can significantly alter soil bacterial community structure, however, the microbiomes within macro-species, such as earthworms, are stable in comparison (Swart *et al.*, 2020). Host-associated microbiomes, although normally very stable, can be susceptible to changes when under stress, for example, changes in environmental conditions such as salinity, pH, climate change (Thurber *et al.*, 2009; Fan *et al.*, 2013; Lokmer and Wegner, 2015; Saha *et al.*, 2020), exposure to pollutants (Kraemer, Ramachandran

and Perron, 2019; Mittal *et al.*, 2019; Jurelevicius *et al.*, 2021; Quillaguamán *et al.*, 2021), and the quality of their host (Case *et al.*, 2011; Saha *et al.*, 2020).

There is limited literature on the effect of anti-fouling biocides, specifically on host microbiomes, with the majority of literature focusing on environmental microbial communities (i.e. biofilms) and the effects of biocides and heavy metals in sediments. In general, the presence of heavy metals such as copper, zinc, cadmium and lead generally demonstrate negative correlations with total prokaryotic cell numbers as well as reduced diversity (Nogales *et al.*, 2011). Some studies have looked at the effect of anti-fouling paint on biofilm community composition. Although specific species identified vary from study to study (due to natural environmental variability, different treatments, location, season etc.), the same patterns repeat themselves. Overall cell abundance and diversity are reduced in biocidal treatments compared to controls or non-biocidal coatings (von Ammon *et al.*, 2018; Winfield *et al.*, 2018; Catao *et al.*, 2021; Papadatou *et al.*, 2021). It's also important to note that the community composition shifts that are seen, are usually defined by only a few taxa. In Papadatou *et al.*, (2021), the overall core community of OTUs (Operational Taxonomic Unit) shared between all samples consisted of a diverse group of genera (60), but only 4 of which contributed any significant abundance (1%) to the core community. Although the biocidal treatment demonstrated less diversity overall, it also had 18 unique genera, 9 of which contributed with 1% abundance, demonstrating a distinct community make up. This community shift was also seen in Winfield *et al.*, (2018) who found an overall reduction in abundance and diversity, but more unique genera in the biocidal treatment compared to the (foul release) non-biocidal coatings. These two studies also used some of the same commercially available coatings as each other (Intersmooth® 7460HS and Intersleek® 900). Interestingly, Flach *et al.*, (2017) and von Ammon *et al.*, (2018) both found Gammaproteobacteria dominated on their anti-fouling coatings. These bacteria have been reported to be particularly tolerant to copper-based coatings and associated with stressed environments. The shifts in community composition on these anti-fouling coatings have been linked to metal- and/or biocide-tolerant taxa (von Ammon *et al.*, 2018; Catao *et al.*, 2021; Papadatou *et al.*, 2021).

1.2 The Importance of the Microbiome

The microbiome is defined as "a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties. The

microbiome not only refers to the microorganisms involved but also encompass their theatre of activity, which results in the formation of specific ecological niches" (Berg *et al.*, 2020). Microbiomes play a crucial symbiotic role in maintaining the health and functioning of individual organisms (Sehnal *et al.*, 2021) as well as at an ecosystem scale (Wilkins *et al.*, 2019). The term holobiont encompasses the role and dynamics of the microbiome and their environment/host, and is considered as a synergistic ecological unit. By assessing holobionts as a whole rather than as just 'the host' or 'microbiome', we can better understand the complex relationships between the two as well as better assess their wider ecosystem function (Petersen and Osvatic, 2018; Trevathan-Tackett *et al.*, 2019).

Host-associated microbiomes can change with the host's developmental life stages, (Aldred and Nelson, 2019). However, for some species, their associated microbes are fundamental to development. In the moon jellyfish *Aurelia aurita*, development was completely prevented in the early life stages (the ephyrae) in those reared in sterile conditions. It was demonstrated that this wasn't solely an effect of being 'sterile', as if allowed for microbial recolonisation, host development was able to progress (Weiland-Bräuer *et al.*, 2020). Host-microbe interactions also include defence mechanisms against pathogens, usually through the production of antimicrobial substances (Shnit-Orland and Kushmaro, 2009; Shnit-Orland, Sivan and Kushmaro, 2012).

Microbiomes and their functioning will have further interactions with the surrounding environment and/or their host. For example, Pita *et al.*, (2018) describes the relationship between the sponge holobiont, the host sponge and the environment. Functionally, microbiomes can have major roles in nutrient cycling, processing compounds that the host can not create itself, and fixing nutrients such as carbon, nitrogen and sulphur (Labbate *et al.*, 2016; Pita *et al.*, 2018; Pogoreutz *et al.*, 2022; Sampaio *et al.*, 2022).

However influences such as climate change, environmental stress and pollutants can disrupt the normal functioning (Mittal *et al.*, 2019; Wilkins *et al.*, 2019; Quillaguamán *et al.*, 2021). In the context of climate change and thermal stress, it has been demonstrated that microbial community shift and functionality can happen in correspondence to temperature change (Lokmer and Wegner, 2015; van de Water *et al.*, 2018; Vargas, Leiva and Wörheide, 2021). Climate-related community shifts can

have repercussions for biogeochemical cycling and carbon flow, which in turn may exacerbate or attenuate climate change (Naylor *et al.*, 2020). In corals, temperature alters the metabolic potential of *Vibrio* spp. associated with the coral, thus providing an avenue for opportunistic pathogenesis (Thurber *et al.*, 2009).

Pollutants (biocides, heavy metals, leachates inclusive) can have dramatic effects on community composition and function in some microbiomes. Focardi *et al.*, (2022) found that leachates from plastics had significant impacts on marine microbiome composition. Specifically, microbial primary producers, which compromise the base of the marine food web, showed significant declines in photosynthetic efficiency, diversity and abundance when exposed to these plastic leachates. This exposure favoured fast-adapting, motile organisms and exhibited enrichment in genes associated with pathogenicity and specific metabolic capabilities. Overall, this can potentially have significant effects on trophodynamics and biogeochemical cycling within the environment (Focardi *et al.*, 2022).

Microbiomes, in general, can be relatively stable, and even with external influences, may undertake some form of self-regulation (Posadas *et al.*, 2022; Vargas *et al.*, 2023). Changes in community composition does not always change the functional capabilities of the microbiome, with shifts still able to undertake the required functional capabilities. For example in Pereira, Pilz-Junior and Corção, (2021), bacterial communities treated with biocides in oil fields, there were important differences in terms of taxonomy, however, functional characterisation remained very similar. Shifts in the microbial communities can also be adaptable to the circumstances. For example, microbiomes from polluted areas in the Red Sea had higher amounts of oil-degrading bacteria and more diverse hydrocarbon-degrading genera, illustrating their adaptability in response to oil pollution events. However, as a ‘side-effect’ of this adaptability, it also introduced higher amounts of pathogens (Mustafa *et al.*, 2016).

Global climate changes, along with localised pressures like pollution, urbanisation, and overuse of natural resources, have significantly altered ecosystems at both microscopic and macroscopic levels. As a result, these changes have led to the rise and spread of microbial diseases, which can adversely affect human health, food security, and essential ecological processes (Hudson and Egan, 2024). Interestingly, our understanding of host-pathogen interactions in marine environments is still behind

that of human and agricultural systems, despite the potentially disastrous impacts of marine diseases (Trevathan-Tackett *et al.*, 2019). Bowley *et al*., (2020) assessed pathogen risks of the microbial communities attached to Marine microplastics due to their potential to act as long-distance transporters of marine "hitchhikers", where human pathogens were identified. However further research would be required to determine their pathogenicity and virulence potential. This study also highlighted the potential of plastics in the marine environment as reservoirs of AMR genes and MRGs.

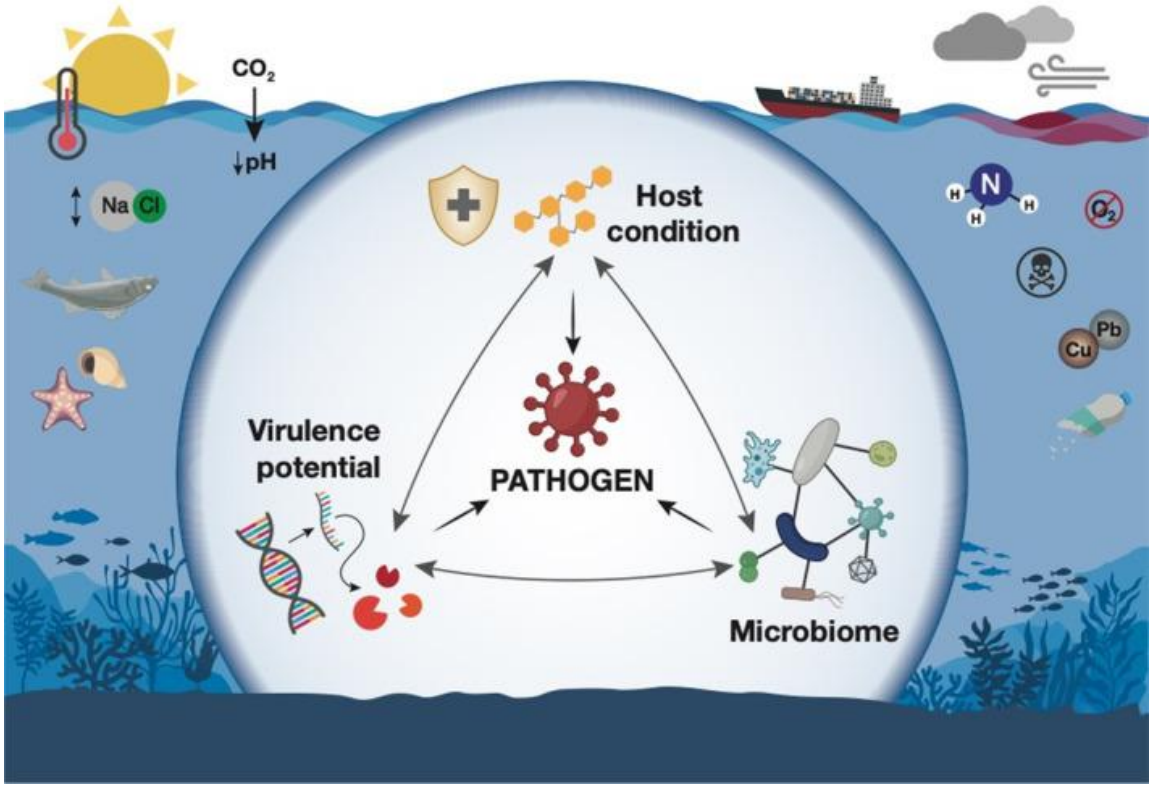


Figure 1.3 Factors influencing the capacity of a microorganism to function as a marine pathogen from Hudson & Egan (2024). Host condition (e.g. immunosuppression), virulence potential and interactions with/between the host microbial community can each play a role in the pathogenic potential of marine microorganisms. It is predicted that under 'normal' conditions, there is a balance between these variables, which suppresses the virulence potential and growth of pathogens. However, the presence of exogenous stressors, such as temperature, pH, salinity, pollutants, eutrophication, hypoxia, invasive species and anthropogenic activity, can have differing effects on host-microbiome-pathogen interactions. These stressors are predicted to weaken host health and microbial community structure, while augmenting (directly or indirectly) the virulence of organisms, which may collectively function to promote pathogenesis.

1.2.1 The Resist-ome and Antimicrobial Resistance (AMR) in the Environment

The microbial communities that thrive on anti-fouling coatings will favour those that demonstrate metal and biocide resistance, however as a consequence; this also selects for other resistance genes, such as anti-microbial resistance (Scientific

Committee on Emerging and Newly-Identified Health Risks, 2007; Kampf, 2019). Resistance phenotypes can manifest themselves through three key mechanisms: (i) co-resistance: which occurs when genes are physically located together on the same genetic element such as a plasmid, transposon or integron (Chapman, 2003) (ii) cross-resistance: single genetic elements/biochemical system that can confer resistance to both antibiotics and metals (e.g. efflux systems). (iii) co-regulation: whereby various regulatory systems are transcriptionally linked, thus, exposure to one toxicant can form resistance responses to another (metals and antibiotics) (Baker-Austin *et al.*, 2006).
As availability to sequencing becomes cheaper and more accessible, there are increasingly more studies looking at the co-occurrence of genes within metagenomes. Primarily, the focus has been on pollutants and heavy metals within the environment, which has resulted in severe effects on aquatic ecosystems, and poses serious risk to human health. Jurelevicius *et al.*, (2021) assessed rates of pathogens in marine environments based on levels of anthropogenic impact. There was a higher diversity of potentially pathogenic species in the 'high' impact areas, as well as higher rates of AMR genes. Similarly, Li *et al.*, (2021) found significant correlations between metal pollutants with microbial community composition as well as antimicrobial and resistance genes in river sediments, the results of which are echoed by similar studies (Mittal *et al.*, 2019; Rodgers *et al.*, 2019; Thomas *et al.*, 2020; Quillaguamán *et al.*, 2021).

Marine biofilms serve as excellent indicators of overall water quality and the ecological health of aquatic systems (Sentenac *et al.*, 2022; Ramljak *et al.*, 2024). Therefore, it is particularly important to evaluate how biofilm communities react to anthropogenic pollution in aquatic environments. This is especially relevant given the increasing presence of chemical compounds, such as metals, personal care products, and medications used in veterinary and human medicine, which are introduced into water bodies primarily through wastewater treatment effluents and agricultural runoff (Pruden *et al.*, 2021; Liguori *et al.*, 2022). Chronic exposure is therefore likely to create 'hotspots' of AMR and other resistance genes (Balcázar, Subirats and Borrego, 2015)

However, when it comes to anti-fouling coatings, the availability of the biocidal ingredients (metallic and non-metallic) are generally tied up within the coatings (although leaching and erosion does occur, which can accumulate within the environment). Flach *et al.*, (2017) has established a correlation between AMR in

biofilms and anti-fouling coatings specifically. They concluded that the paint did not enrich known mobile antibiotic resistance genes within the microbial biofilms, but instead selected for RND efflux systems and genes involved in mobilization of DNA. The selection pressure from the paints co-selected for resistant bacteria, favouring species and strains carrying genes that demonstrate cross-resistance.

Its important to note, that generally resistance to biocides specifically is not very common due to the lack of known detoxifying enzymes and target multiplicity within the cell. Resistance to biocides usually occurs as a result of changes in cell envelope permeability or enhanced cellular efflux. The latter plays an important role in biocide resistance, hence the close relationship between biocide, metal and antibiotic resistance (Goudarzi and Navidinia, 2019).

This is of particular concern due to the apparent increased selection pressure on antimicrobial resistance. AMR is of growing and heightened concern, as highlighted by the World Health Organization (WHO) as a danger to environmental, animal and human health. It has been estimated that 35,000 people a year in Europe alone, die directly from drug-resistant bacterial infections (WHO-Europe and ECDC, 2022) , while worldwide predictions suggest mortalities of half a million people (Davies *et al.*, 2013). With the recent pandemic, the WHO have expressed concern about the increased use of antibiotics in COVID-19 patients, and the use of disinfectants within our homes, potentially selecting for resistant bacteria and pathogens (Getahun *et al.*, 2020).

The problem with AMR in the environment, lies with the increased prevalence of pathogenic microbes that also carry AMR genes, which are seeing an unprecedented increase due to selective pressure from anthropogenic sources, of which there are many (Singer *et al.*, 2016). Drivers affecting aquatic environment levels of AMR include climate change (MacFadden *et al.*, 2018), waste discharge (anti-microbials from sewage) (Kraemer, Ramachandran and Perron, 2019), pesticides (Nogales *et al.*, 2011), antibiotic use in farming and aquaculture (Guardiola *et al.*, 2012), pollution e.g. heavy metals (Rodgers *et al.*, 2019), and now anti-fouling paints are facing the limelight.

1.3 One Health Approach

The World Health Organization (WHO) has identified Antimicrobial resistance as a serious challenge to address, with AMR already reaching alarming levels in many parts of the world (European Commission, 2017; WHO-Europe and ECDC, 2022). AMR already presents a serious social and economic burden. It is estimated to be responsible for ~35000 deaths per year in the EU alone in 2020 (WHO-Europe and ECDC, 2022), 10,000 more than a decade earlier (*European Centre for Disease Prevention and Control. Joint Report with EMEA: The Bacterial Challange: Time to React*, 2011). Globally, AMR was directly responsible for 1.27 million deaths per year and indirectly contributed to 4.95 million deaths in 2019 (Murray *et al.*, 2022). Inaction is projected to cause millions of deaths globally, estimated to cause more deaths than cancer by 2050 (O'Neill, 2016; Patel *et al.*, 2017).

The One Health approach, as defined by the European Commission is: "a term used to describe a principle which recognises that human and animal health are interconnected, that diseases are transmitted from humans to animals and vice versa and must therefore be tackled in both. The One Health approach also encompasses the environment, another link between humans and animals and likewise a potential source of new resistant microorganisms". This term is globally recognised, having been widely used in the EU and in the 2016 United Nations Political Declaration on AMR (European Commission, 2017).

The role that the environment plays in the spread of AMR is increasingly being acknowledged for its increased contribution and risk of AMR related issues, however, more extensive evidence has been requested by the EU Commission to better understand and inform decision-making (European Commission, 2017). Most AMR surveillance in animals primarily focuses on domestic livestock due to the overuse of antibiotics and our close connections and risk (Kraemer, Ramachandran and Perron, 2019). However AMR surveillance in wildlife, particularly marine wildlife, is a relatively new field and is still being developed. Terrestrial wildlife surveillance focuses on mammalian species such as bears (Brealey *et al.*, 2021), deer, goats (Smoglica *et al.*, 2023), wild boar, and some avian species (Plaza-Rodríguez *et al.*, 2021). In the aquatic and marine environments, efforts have focused on areas of aquaculture and coastal activities (Smith *et al.*, 2015; Light *et al.*, 2022; Environment Agency, 2023), waste water outputs (Kotlarska *et al.*, 2015; Aubertheau *et al.*, 2017; Gevao *et al.*, 2022;

Kvesić *et al.*, 2022) and microbial communities in heavily contaminated areas (Rodgers *et al.*, 2019). Therefore, by addressing the gap in knowledge and assessing the current state of marine microbiomes which have already been anthropogenically impacted, we can determine tools and policies that may help with the restoration and/or mitigation of ecosystem decline and prevent further development of the marine 'resistome' (Peixoto and Voolstra, 2023).

1.4 Summary and Next steps

As identified by the One Health Action Plan and the World Health Organization, there is a lack of environmental and animal microbiome research in relation to anthropogenic impacts and AMR and pathogen prevalence. The current literature for effects of anti-fouling biocides on microbiomes are incredibly limited, even though the use of anti-fouling is used on a global scale, and the proximity humans come into contact with these communities is minute, thus potentially exposing ourselves to reservoirs of unassessed pathogens and antibiotic-resistant microbes (Trevathan-Tackett *et al.*, 2019). Animal-associated microbiomes are also distinct from that of their surrounding environment and behave very differently to the equivalent biofilm on the same substrate.

This study aims to fill some of these knowledge gaps by looking at how biocidal and non-biocidal anti-fouling coatings affect invertebrate microbiome composition and antibiotic resistance profiles. It will also assess microbiome transfer between generations to address potential risks associated with altered microbiome transfer to new generations and, consequently, the wider environment.

Chapter 2: Characterising The Colonising Biofilm Communities on Different Anti-fouling Coatings

2.1 Introduction

In many environments, microorganisms adhere themselves to surfaces, forming a complex matrix of biopolymers (Dobretsov, 2010; Balcázar, Subirats and Borrego, 2015; Kirstein *et al.*, 2018; Angelova *et al.*, 2019). This biofilm matrix can be composed of a single bacterial species; however, it is most commonly formed by a diverse and complex community of microorganisms such as bacteria, algae, fungi and protozoa. These organisms embed themselves in an extracellular matrix of polysaccharides supplemented with exudates and detritus. The type of surface and environmental conditions can, however, impact the biofilm species and, thus, the community composition, biofilm properties and functionality of the fouling community (Flach *et al.*, 2017; Kirstein *et al.*, 2018; Corcoll *et al.*, 2019; Roberto *et al.*, 2019; Zhang *et al.*, 2019; Catao *et al.*, 2021).

Anti-fouling coatings traditionally contain biocidal components, which are used to prevent colonisation through toxicity. However, some microbial species are 'resistant' to these biocides, and therefore create unique communities to survive in the presence of biocidal agents (Chen *et al.*, 2013; Kirstein *et al.*, 2018; Papadatou *et al.*, 2021; Zhai *et al.*, 2022). Copper oxide and other copper-based compounds (e.g. copper thiocyanate, dicopper oxide) are the most common and can be found in the majority of anti-fouling coatings, usually accompanied by other booster biocides such as zinc pyrithione (HSE, 2024). Therefore, 'anti-fouling' resistant microbes are usually associated with metal resistance mechanisms (Hobman and Crossman, 2015; Ding *et al.*, 2019; Catao *et al.*, 2021).

It is becoming apparent that microbial communities impacted by anthropogenic influences (e.g. biofilms, sediments, waste water) can act as environmental reservoirs of resistance, forming the 'resistome' (Baker-Austin *et al.*, 2006; Balcázar, Subirats and Borrego, 2015; Pal *et al.*, 2015; Imran, Das and Naik, 2019; Mittal *et al.*, 2019; Roberto *et al.*, 2019; Thomas *et al.*, 2020; Quillaguamán *et al.*, 2021). The relationship between metal resistance and antimicrobial resistance has been strongly correlated through shared mechanisms (Scientific Committee on Emerging and Newly-Identified Health

Risks, 2007; Hobman and Crossman, 2015; Pal *et al.*, 2015). The collection of resistance genes (primarily antibiotic, but can include other resistance genes too) within a set biome or environment forms the resistome. Species that tend to harbour these resistance genes, also tend to include pathogenic groups. It is thus important to evaluate this relationship on anti-fouling coatings, which have the potential to carry these genes and potential pathogens around the globe. Resistome risk refers to the conceptual framework introduced by Martinez *et al*. (2015), in which it is assumed that antibiotic resistance genes (ARGs) 1) confer resistance to antibiotics currently used for therapeutic purposes, 2) are associated with mobile genetic elements (MGEs), and 3) when carried by human pathogens represent the greatest public health risk (Martínez, Coque and Baquero, 2015; Afrin Rumi *et al.*, 2024). Assessments, such as the use of MetaCompare (Oh *et al.*, 2018; Afrin Rumi *et al.*, 2024) have been developed to account for environmental dimensions and challenges of antimicrobial risk assessment, which can be narrowed down to two risk outcomes: ecological resistome risk (ERR) and human health resistome risk (HHRR). The ERR score factors in a wide-ranging array of pathogens and ARGs in order to broadly represent the potential for ARGs to mobilize in a given environment. The HHRR, on the other hand, focuses more specifically on pathogens that are of most acute concern with regard to antibiotic resistant infections in humans (i.e., ESKAPE pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) and Rank I ARGs (Zhang *et al.*, 2021). ESKAPE pathogens have been recognized as World Health Organization (WHO) priority pathogens that exhibit high virulence and antibiotic resistance (WHO, 2017). ESKAPE pathogens have also been shown to be enriched with a specific subset of acquired mobile ARGs in anthropogenically-impacted environments (Zhang *et al.*, 2021).

Other implications of exposure to anti-fouling biocides include changes in community composition, which could lead to a change in the functionality of the biofilm. Bacterial biofilms will produce a range of different compounds, including but not limited to; extracellular polymeric substances (EPS) (Tang and Cooney, 1998), toxins (Semenyuk *et al.*, 2014), and anti-microbials (Wilson, Raftos and Nair, 2011; Goel *et al.*, 2021). Larvae of many marine invertebrates use biofilm components as cues for appropriate settlement sites (Hadfield, 2011; Whalan and Webster, 2014; Dobretsov and Rittschof, 2020), therefore changes in biofilm community (composition and/or density), could

impact any successional macrofouling (Huang and Hadfield, 2003). Established biofilms may also influence the microbiome acquisition of any settling organisms (Aldred and Nelson, 2019). Knowledge of marine biofilm components on fouling control coatings will serve as a guide for future investigations of marine microfouling (Papadatou *et al.*, 2021). Common and rare taxa within a community will differ with location and substrate type, with specific species and their exudates often acting as indicators of suitable settlement substrates for macro-foulers (Hadfield, 2011; Ganesan *et al.*, 2012), or from a monitoring perspective, certain species may act as environmental indicators of condition or anthropogenic impact (Ramljak *et al.*, 2024).

This study aimed to characterise the colonising bacterial biofilm on different anti-fouling paint systems, using next generation 16S sequencing complemented by predictive functional analysis and exploratory shotgun sequencing to identify whether resistance genes are enriched in biocidal vs non-biocidal coatings.

2.2 Methods

2.2.1 Settlement Boards and Study Area

Settlement boards were made using 3mm polyvinyl chloride (PVC) panels (60x40cm) (Plastics Ltd, North Shields UK), which were sanded (120 grade) and primed for paint application. Six paints were used in this study, including an epoxy primer control (Figure 2.1, Table 2.1). Appropriate primers were applied in two layers, before applying the final two top coats (Coppercoat needed six layers as standard). The paints were as follows: Hempel Light Primer on its own (non-biocidal), International Primocon with International Trilux 33 (biocidal), International Primocon with International VC Offshore EU (biocidal), Hempel Underwater Primer with Hempel Ecopower Cruise (non-biocidal), Hempel Light Primer with Coppercoat (biocidal), Hempel Light Primer followed by Silic One Tie Coat and two coats of Silic One Top Coat (non-biocidal). Panels were then deployed at Newcastle University's Blyth Marine Station (07/09/2021) coordinates: 55°07'32.1"N 1°29'51.5"W and 55°07'32.2"N 1°29'51.3"W.

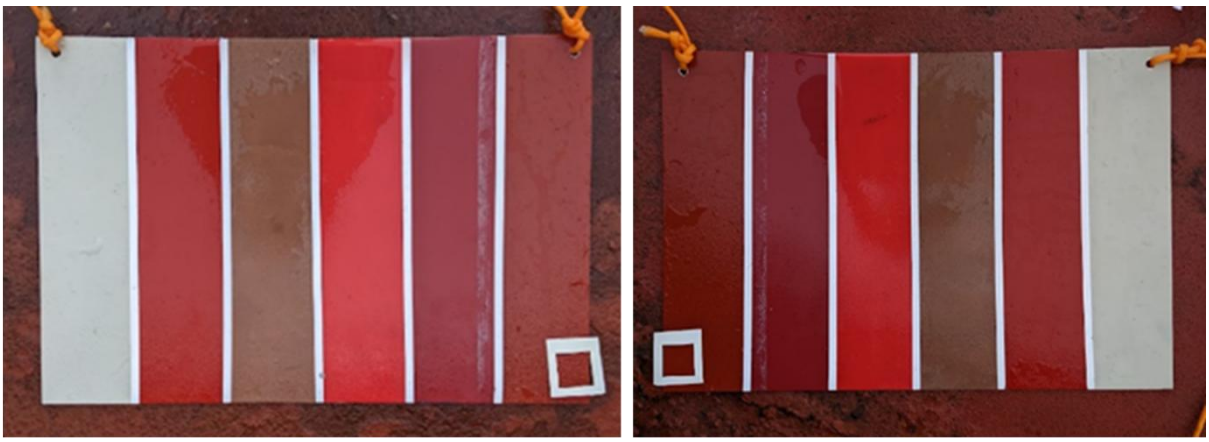


Figure 2.1 Panel 'A' (left) and panel 'B' (right). Panel A paint order: Hempel epoxy Primer, Hempel Ecopower Cruise, Coppercoat, International Trilux 33, International VC Offshore EU, Hempel Silic One foul release. Panel B is in the reverse order. The small white square is the quadrat used for sampling (4x4cm).

Table 2.1 Table of percentages of active ingredients in the coatings tested in this study. Preventol A4S concentration is unavailable to the public as is part of International's secret formulation

Active ingredient	VC Offshore	Trilux33	Coppercoat	Silic One	Ecopower	Primer
Copper thiocyanate	-	≥10 - ≤15	-	-	-	-
Pyrithione zinc	-	≤3.5	-	-	-	-
Copper oxide	20	-	95	-	-	-
Preventol A4S	undisclosed	undisclosed				

2.2.2 Sample Collection

Samples were collected at time: 0, 1, 3, 7, 14, 21, 29 and 56 days. Two swabs per surface were collected using Novacyt Microgen swabs (Fisher Scientific, UK) with care taken not to resample a previously sampled area during this study using a 4cm x 4cm quadrat. A one-litre water sample was also collected at time intervals, along with environmental data using a HORIBA sensor (HORIBA Ltd, Japan) (Supplementary Figure 2.1). All samples were kept in an ice box and stored at -80^{o}C on arrival to the laboratory. Water samples were vacuum filtered onto sterile 0.2µm mixed nitrocellulose filter membranes (Merck Millipore, USA) and stored at -20^{o}C for later analysis.

2.2.3 DNA Isolation

DNA was extracted using the DNeasy PowerLyzer PowerSoil kit (QIAGEN). Each biofilm swab was placed into a PowerSoil bead tube using sterile forceps. QIAGEN's protocol was followed according to the manufacturer's instructions (DNeasy PowerLyzer PowerSoil kit august 2016), with a few alterations: An additional lysis step at 60°C for 10 minutes was added and, post bead beating, swabs were centrifuged in an adapted extraction tube (filter removed) to obtain trapped supernatant. Prior to elution, an additional wash step using ice-cold 80% ethanol was also added. Samples were eluted in a total volume of 70µl elution buffer. Samples were quantified using a Tecan NanoQuant plate reader (TECAN, Switzerland).

2.2.4 Metagenomic Sequencing

2.2.4.1 PCR Amplification and Amplicon Sequencing of 16S rRNA

PCR was performed using the Earth Microbiome Project primer set 515F–806R, which targets the V4 region of the 16S SSU rRNA (FWD:GTGYCAGCMGCCGCGGTAA (Parada, Needham and Fuhrman, 2016); REV:GGACTACNVGGGTWTCTAAT (Apprill *et al.*, 2015). Using the pre-barcoded primers (Integrated DNA Technology), a PCR was run under the following conditions: 94°C for 3 mins, followed by 35 cycles of: 94°C for 45 secs, 50°C for 60 secs, 72°C for 90 secs, then a final extension period of 72°C for 10 mins. PCR was carried out using 0.8x KAPA 2G Robust Taq polymerase (Roche), 0.2µM of each primer and 1µl of DNA. The PCR products were measured using PicoGreen (Invitrogen) on a Spark plate reader (TECAN), then normalised to a concentration of 50nmol/µl. The library was made by pooling 5µl from each sample together prior to clean up using AMPure XP (Beckman Coulter), followed by two washes using 80% ethanol. The library was resuspended in molecular grade water. Library concentration was remeasured using the high-sensitivity DNA kit (Invitrogen) using the Qubit 4.0 (Invitrogen), and the product size was checked using the high-sensitivity DNA kit (Agilent) on the Agilent Bioanalyzer 2100. The library was dissociated into single strand DNA using an equal volume of 0.2N sodium hydroxide which was stopped after 5 minutes using 400nM Tris pH 8.0 buffer. The library was then diluted to 20pMol with Illumina hybridisation buffer before being spiked with 20% v/v PhiX (phage lamda DNA) as in internal quality control. The final library was loaded into the Illumina MiSeq v2 500 cycle reagent cartridge.

2.2.4.2 Shotgun Sequencing

DNA was diluted to 10ng/µl or where unavailable a total volume of 1.3 µl was added directly without dilution. The library was prepared using NEBNext Ultra II FS DNA kit using a scaled-down protocol (Haichao Li *et al.*, 2019). The library was made by pooling 5µl from each sample together prior to clean up using AMPure XP (Beckman Coulter), followed by two washes using 80% ethanol. The library was resuspended in molecular-grade water. Library concentration was remeasured using the high-sensitivity DNA kit (Invitrogen) using the Qubit 4.0 (Invitrogen), and the product size was checked using the high-sensitivity DNA kit (Agilent) on the Agilent Bioanalyzer 2100. The library was diluted to 0.75nM and denatured with 0.2N NaOH giving a final library concentration of 150pM and sequenced on an Illumina NovaSeq 6000 SP500 reagent cartridge.

2.2.5 Bioinformatic and Statistical Analysis

2.2.5.1 Qiime2 Pipeline for 16S rRNA Analysis

Pre-demultiplexed fastq files were processed using the Qiime2 package, version 2023.2 (Figure 2.2) (Bolyen *et al.*, 2019). Sequences were quality checked using FastQC (Andrews, 2010), trimmed and denoised using DADA2 (Callahan *et al.*, 2016) with reads truncated to f-243 r-208 based on 25th percentile above a quality score of 30, and chimeras removed. Features with very low abundance, below 10 reads, were removed. ASVs (Amplicon Sequence Variants) were created based on the remaining reads and assigned taxonomies using the Silva (version 138) (Quast *et al.*, 2013) and Greengenes2 (McDonald *et al.*, 2023) databases. Following taxonomic assignment, unassigned, eukaryotic, mitochondria, and chloroplast hits were removed (Supplementary Table 2.1). A phylogenetic tree was created using Fasttree for downstream analysis. Feature tables were exported for analysis and visualisation performed in R (version 4.3.1,R Core Team, 2023).

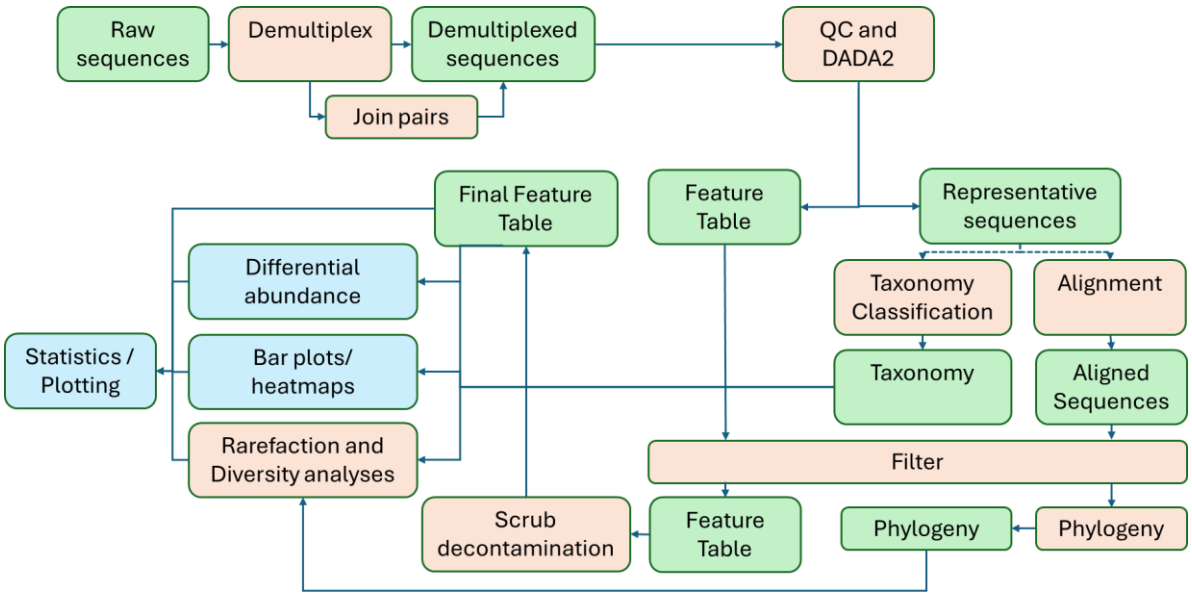


Figure 2.2 Workflow overview adapted from the Qiime2 workflow. Green boxes represent data formats, orange boxes represent data processing steps, blue boxes represent outputs.

2.2.5.2 16S Community Statistical Analysis and Visualisation

Potential contamination identified in the negative controls was removed using the package SCRuB (Austin *et al.*, 2023). For diversity metric analysis, samples were rarefied to 14445 reads based on the lowest sample count. For 'mature' biofilm samples (more than 21 days old), a higher rarefaction was conducted to 30195 reads. Rarefaction and alpha diversity metrics were conducted using the community ecology package Vegan v2.6-6.1 (Oksanen *et al.*, 2024) and Phyloseq v1.44 (McMurdie and Holmes, 2013). Pairwise comparisons were conducted using the Wilcoxon rank sum exact test on alpha diversity metrics. Community composition was transformed from counts to relative abundance for community visualisation. Beta diversity and associated distance plots were conducted through Phyloseq (McMurdie and Holmes, 2013). Differential abundance analysis was conducted using DESeq2 v1.42 on non-rarefied data (Love, Huber and Anders, 2014).

2.2.5.3 Screening of Predicted Functional Profiles with PICRUSt2

Using the ASVs with Greengenes2 assigned taxonomies, the predicted functional characteristics of the biofilm bacterial community were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) via the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) package (Douglas *et al.*, 2020). PICRUSt2 predicts the function of the bacterial community based on the proportion of marker genes present in each sample. Downstream analyses and

visualisations were conducted using Microbiome Analyst 2.0 (Dhariwal *et al.*, 2017; Chong *et al.*, 2020; Lu *et al.*, 2023) and STAMP v2.1.3 (Parks *et al.*, 2014).

2.2.5.4 Shotgun Metagenomic Analyses: Calculations of ARG and MetaCompare2.0 Risk Scores

Raw shotgun samples were subject to FastQC and quality trimming using Trim Galore v0.6.5 (default parameters as trimmed reads, quality score of 20 and stringency of 4) (Krueger, 2017). Taxonomic assignment and abundance of shotgun reads were produced using Kraken2 v2.13 (Wood, Lu and Langmead, 2019) and Bracken v2.9 (Lu *et al.*, 2017) pipelines. Contig assemblies were made using metaSPAdes (Nurk *et al.*, 2017) and then input through Abricate v0.6.10 (Seeman, 2020) utilising the MEGARes (Doster *et al.*, 2020), RESfinder (Florensa *et al.*, 2022), VFDB (Chen *et al.*, 2016) and NCBI AMRFinderPlus database (Feldgarden *et al.*, 2019, 2021) to detect antimicrobial resistance (AMR) gene presence.

Quality trimmed and sample merged FASTQ files were run through the ARGs-OAP v2 pipeline (Yin *et al.*, 2018) to determine ARG abundance normalised by 16S rRNA gene copies. The pipeline uses a BLASTX approach by searching the combined ARG databases of CARD (The Comprehensive Antibiotic Resistance Database) (Alcock *et al.*, 2023) and ResFinder (Florensa *et al.*, 2022).

Ecological and human health resistome risk scores were calculated using MetaCompare 2.0 (Figure 2.3) (Afrin Rumi *et al.*, 2024). Assemblies for MetaCompare 2.0 were generated using three assembly methods: metaSPAdes (Nurk *et al.*, 2017), IDBA-UD (Peng *et al.*, 2012) and MEGAHIT (Li *et al.*, 2015).

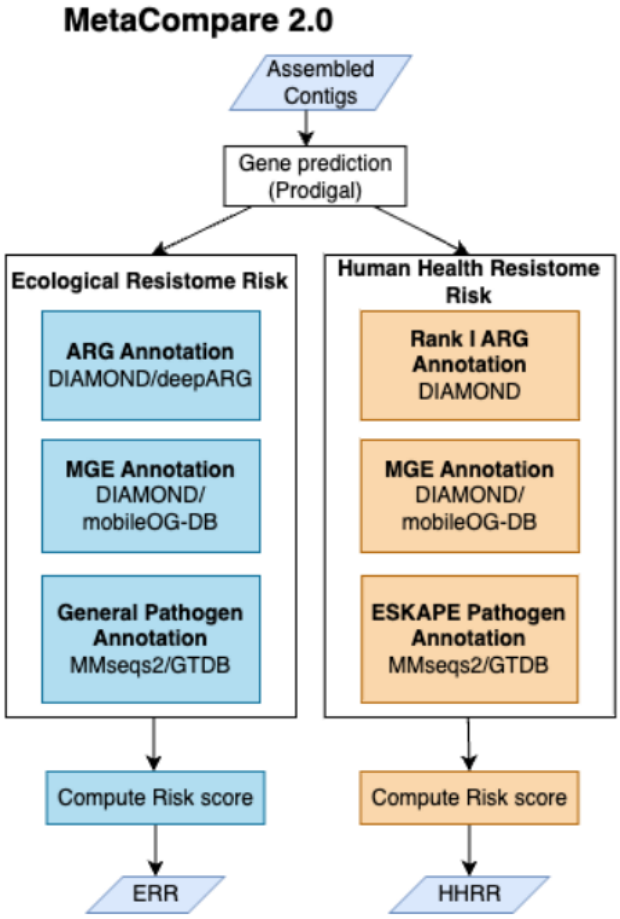


Figure 2.3 Basic pipeline overview of MetaCompare2.0 (from Afrin Rumi et al., 2024). The ecological resistome risk (ERR) evaluates a broad array of both known and putative ARGs, their co-occurrence with MGEs, and a full range of human bacterial pathogens to capture their probable contribution to the proliferation of antibiotic resistance in corresponding environments. The human health resistome risk (HHRR) focuses on a narrower set of ARGs, defined by (Zhang *et al.*, 2021) *as Rank I ARGs, that are: 1) demonstrated to be enriched in "human-associated" environments, 2) mobile (carried by MGE), and 3) can be carried by ESKAPE pathogens.*

2.3 Results

2.3.1 16S Sequencing Data and Rarefaction

A total of 3,475,413 reads were obtained from all sample libraries after quality filtering steps were performed. A total of 5070 ASV's were identified using the SILVA database v138. After subsampling to the level of the library containing the fewest reads (Day-3-B-Trilux = 14445), rarefaction analysis demonstrated curve plateau at the rarefaction point (Figure 2.4).

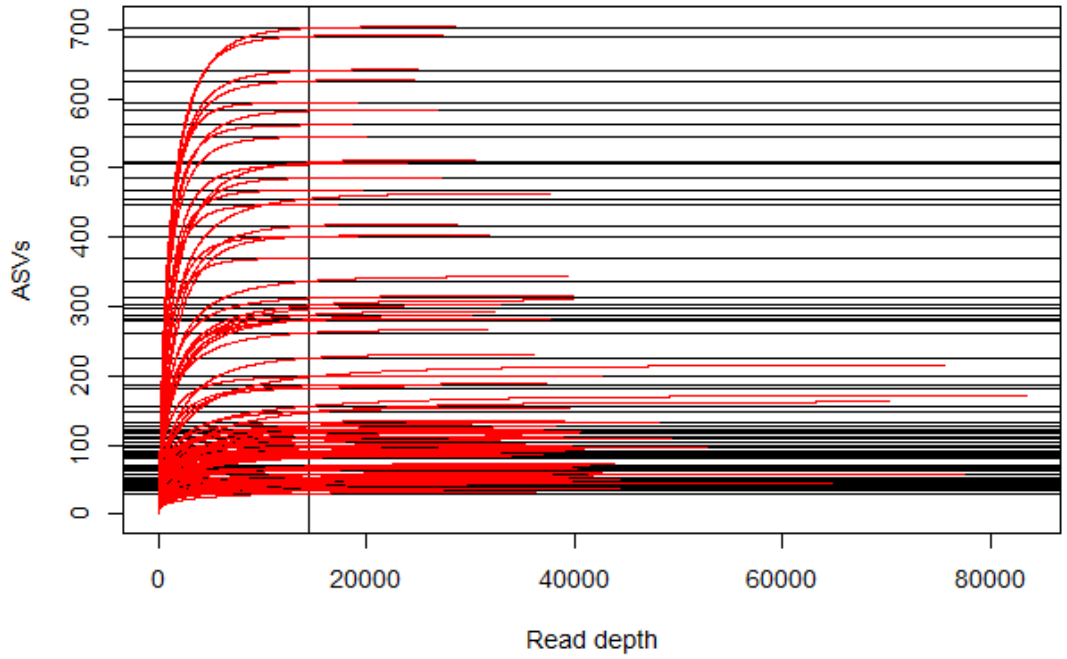


Figure 2.4 Rarefaction curves of all biofilm samples based on observed species found. Rarefaction cut-off at 14445 is seen past the plateau point of all samples, indicating sufficient sampling depth.

2.3.2 Unique ASVs

When all samples from all time scales were compared, there were 5070 ASVs in total with 7.14% shared among treatments and the surrounding seawater. Of the total ASVs, 27.73% were shared between biocidal and non-biocidal treatments (Figure 2.5B). Biocidal and non-biocidal treatments had similar totals of ASVs, but had large portions of treatment-unique ASVs (Figure 2.5A and C). The biocidal treatments contained 32.3% unique ASVs, while non-biocidal contained 30.4% (Figure 2.5B).

For this comparison, samples from day 21 onwards with more 'established/mature' biofilms were used to take into consideration the high variability of early biofilm formation. Across all treatments, a total of 1664 unique ASVs were identified, of which only 4.75% were shared across all categories (biocidal, non-biocidal and seawater), or 22.87% between biocidal and non-biocidal treatments, demonstrating shared and exclusive component taxa between the treatments (Figure 2.6B).

The non-biocidal Primer coating had the most unique ASVs overall (25.12%), followed by: Coppercoat (8.65%), Ecopower (8.59%), Silic One (8.35%), VC Offshore (8.11%) and Trilux (2.34%) (Figure 2.6A and C). Although the primer coating biofilm had the highest number of unique ASVs, they represented only 0.6% of the total community, whereas the Coppercoat biofilm had the highest representation of unique ASVs at 2.6% of the total community (Supplementary Figure 2.2)

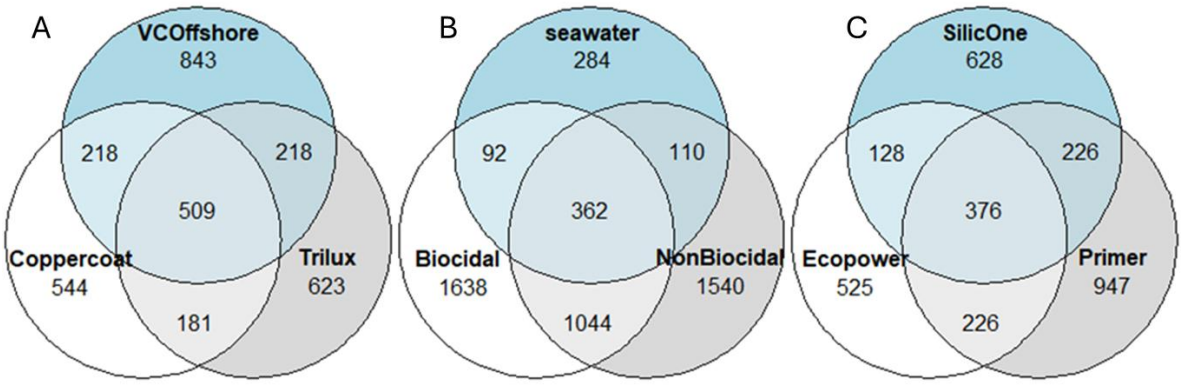


Figure 2.5. Unique ASV counts from all ages of biofilms (A) ASV counts from individual biocidal coating treatments. B) ASV counts from grouped biocidal, nonbiocidal and surrounding seawater. C) ASV counts from individual biocidal coating treatments.

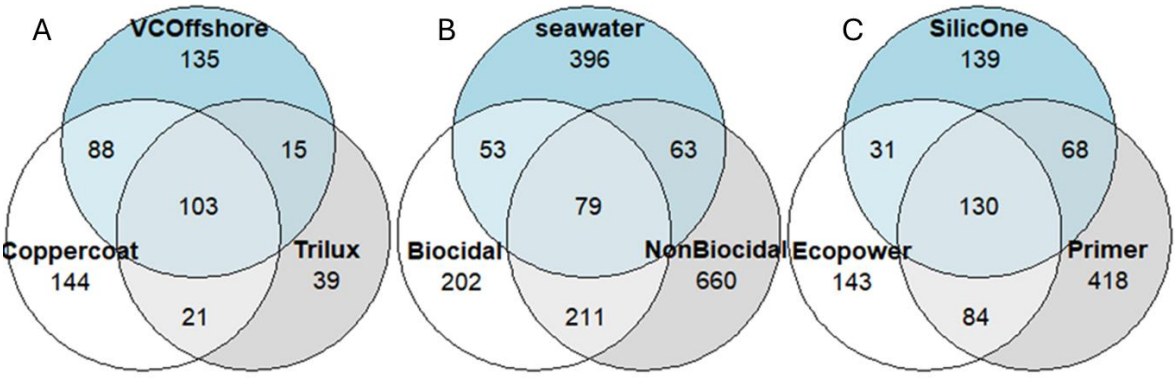


Figure 2.6 Unique ASVs from 'mature' biofilms only (day 21 onwards) A) ASV counts from individual biocidal coating treatments. B) ASV counts from grouped biocidal, nonbiocidal and surrounding seawater. C) ASV counts from individual biocidal coating treatments.

2.3.3 Biofilm Community Changes Over Time

2.3.3.1 Alpha Diversity

Shannon diversity and Pileou's evenness index dropped rapidly between day 1 and day 7 then, as the biofilm developed, diversity steadily increased and levelled off over time (Supplementary Figure 2.3). All tested alpha diversity metrics remained lower compared to the initial submersion. The Primer coating generally exhibited higher diversity (Observed features, Chao, ACE and Fisher) compared to the other treatments over time. In contrast, VC Offshore and Trilux (the biocidal coatings) exhibited lower levels of diversity. The initial drop in diversity between early and 'mid-aged' biofilms appeared to be slightly larger in the biocidal treatments, although not significantly different (Figure 2.7).

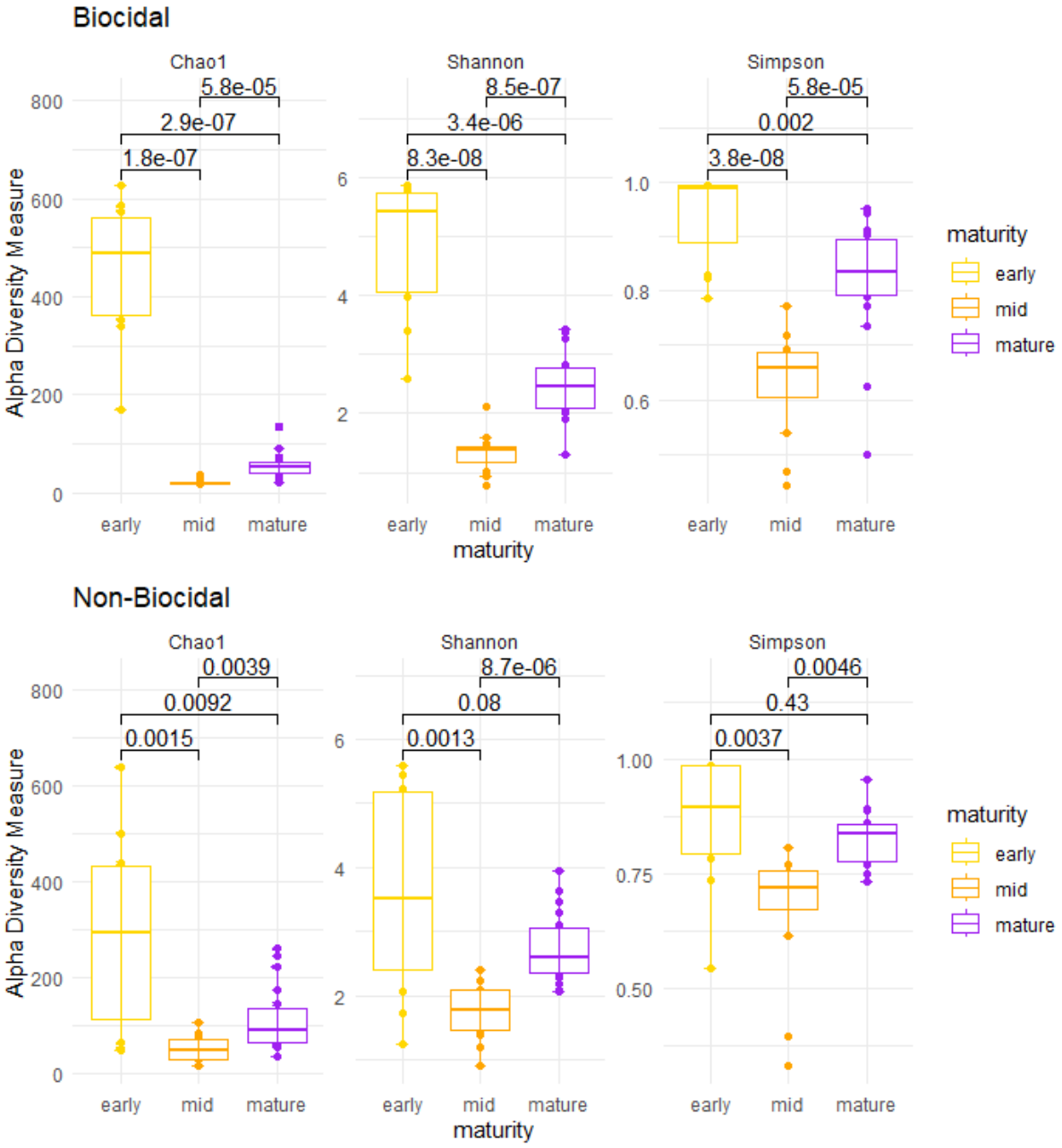


Figure 2.7 Alpha diversity for Eveness (Simpson), Diversity (Shannon) and Richness (Chao1) of maturity levels by Biocidal category. Maturity is categorised as early: days 1 & 3, mid: days 7 & 14, and mature: days 21, 29 and 56. pairwise t-test indicates significant differences in alpha diversity between maturity classes, specifically between early and mature biofilms.

2.3.3.2 Beta Diversity

Using Bray-Curtis dissimilarity and Uni-frac on PCoA plots, there are plot dimensional shifts in the diversity of the biofilm community over time (Figure 2.8). Although time points overlap, those at early stages do not overlap with those at mature stages, demonstrating a clear community shift as the biofilm develops, with the 'mid-aged' biofilms demonstrating intermediary values.

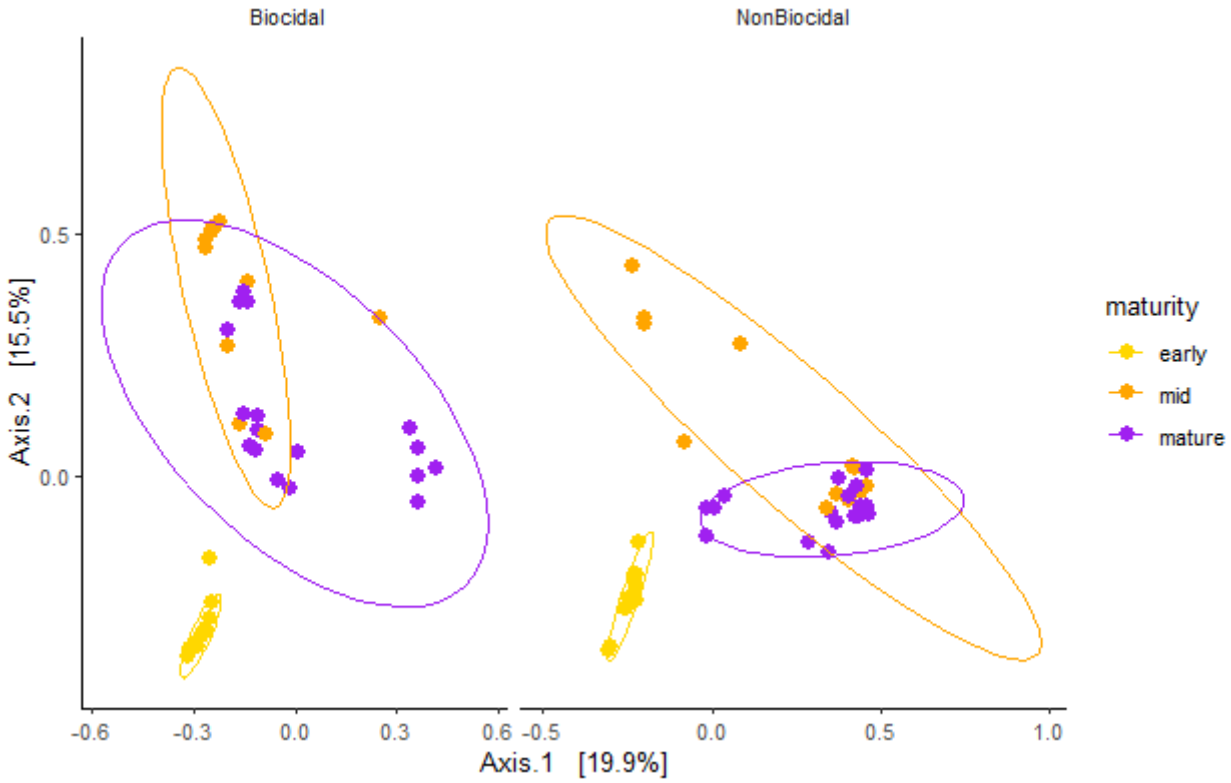
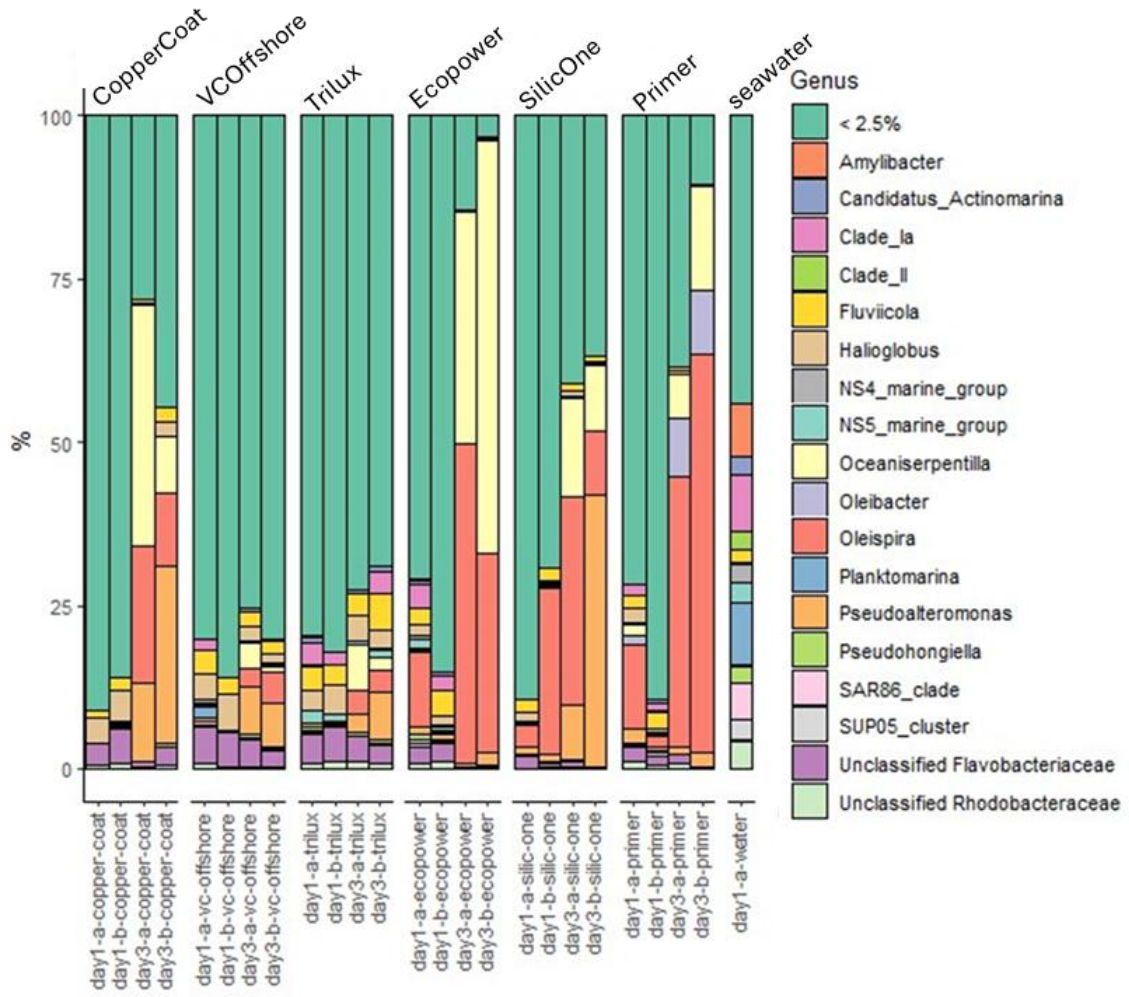


Figure 2.8 Bray-Curtis dissimilarity beta diversity PCoA plots coloured by time progression (maturity) by biocidal and nonbiocidal coating treatments.

2.3.4 Early Colonisers Community Composition

The taxa present on the coatings from day 1 were compositionally different from the surrounding seawater and continued to shift after day 3 (Figure 2.9). The large '<2.5%' category suggests a larger number of individual taxa making small contributions to the overall community composition. After day 3, there is a shift in the dominant taxa, primarily: *Alteromonas* (unclassified sp), *Oceaniserpentilla* (unclassified and *O. haliotis*), *Oleispara* (unclassified sp), and *Pseudoalteromonas* (unclassified sp) across samples. The biocidal Trilux and VC Offshore coatings have a lower relative abundance of dominant taxa at day 3 compared to the other coatings (Figure 2.9).



827

828 *Figure 2.9 Genus community composition of early colonisers (day 1 and 3) across the*
829 *coating treatments and replicates.*

830 ### 2.3.5 Mature Biofilm Community Composition

831 #### *2.3.5.1 Alpha Diversity by Coating/Treatment*

832 Diversity metrics from each coating overlap in 'mature' biofilms of 21 days and older.
833 There is no significant difference between specific coatings (Supplementary Table 2.3).
834 However, there are significant differences when coatings are grouped by biocidal
835 category (biocidal vs non-biocidal coatings) (Figure 2.10) in Observed and Chao1,
836 metrics, but not in Shannon diveristy (Supplementary Table 2.2).

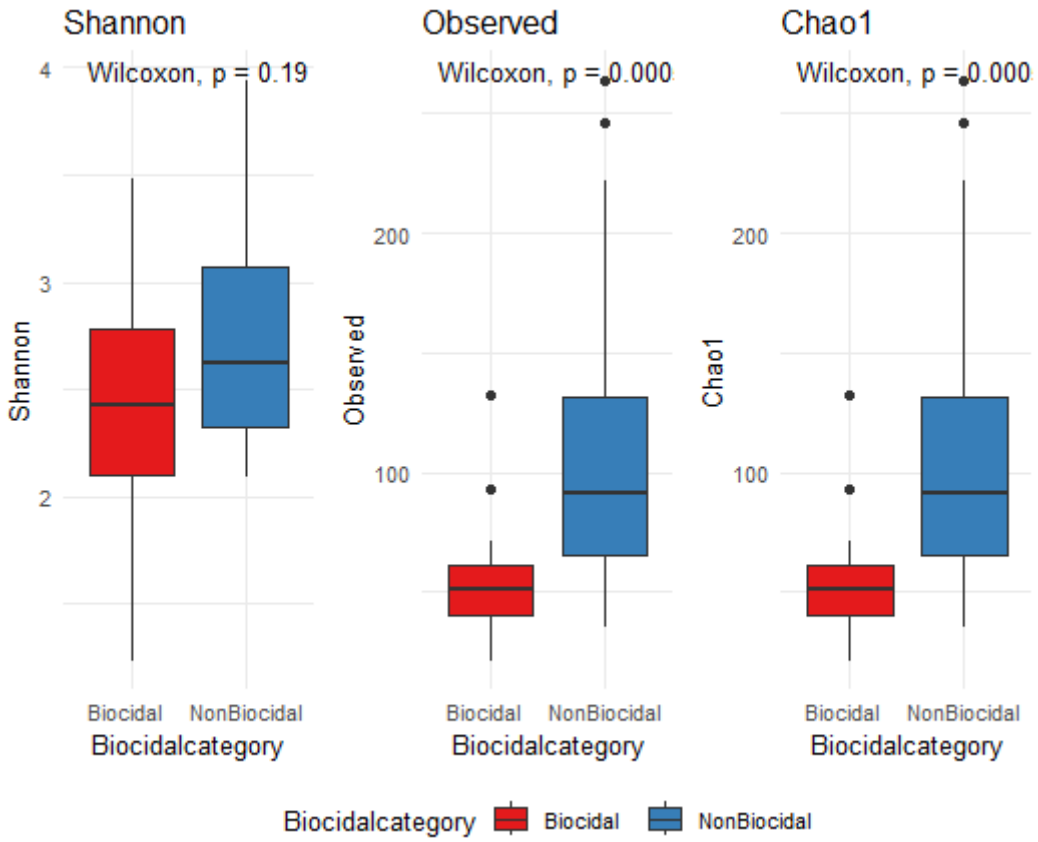


Figure 2.10 Alpha diversity metrics between biocidal and nonbiocidal coatings from day 21 onwards. Shannon Wilcoxon rank: W=120, p=0.19, Observed Wilcoxon rank: W=52, p=<0.001, Chao1 Wilcoxon rank: W=52, p=<0.001.

2.3.5.2 Beta Diversity Changes Between Biocidal and Non Biocidal Treatments

There were evident differences between biocidal and non-biocidal samples using beta diversity metrics (Figure 2.11), indicating distinct community compositions between the two biocidal treatment types but not between individual coatings (Bray-Curtis) (Figure 2.11). Coppercoat and VC Offshore communities showed similar clustering (Figure 2.11), which both contain copper filings. The biocidal Trilux coating demonstrated a high degree of overlap with the non-biocidal coatings (Figure 2.11).

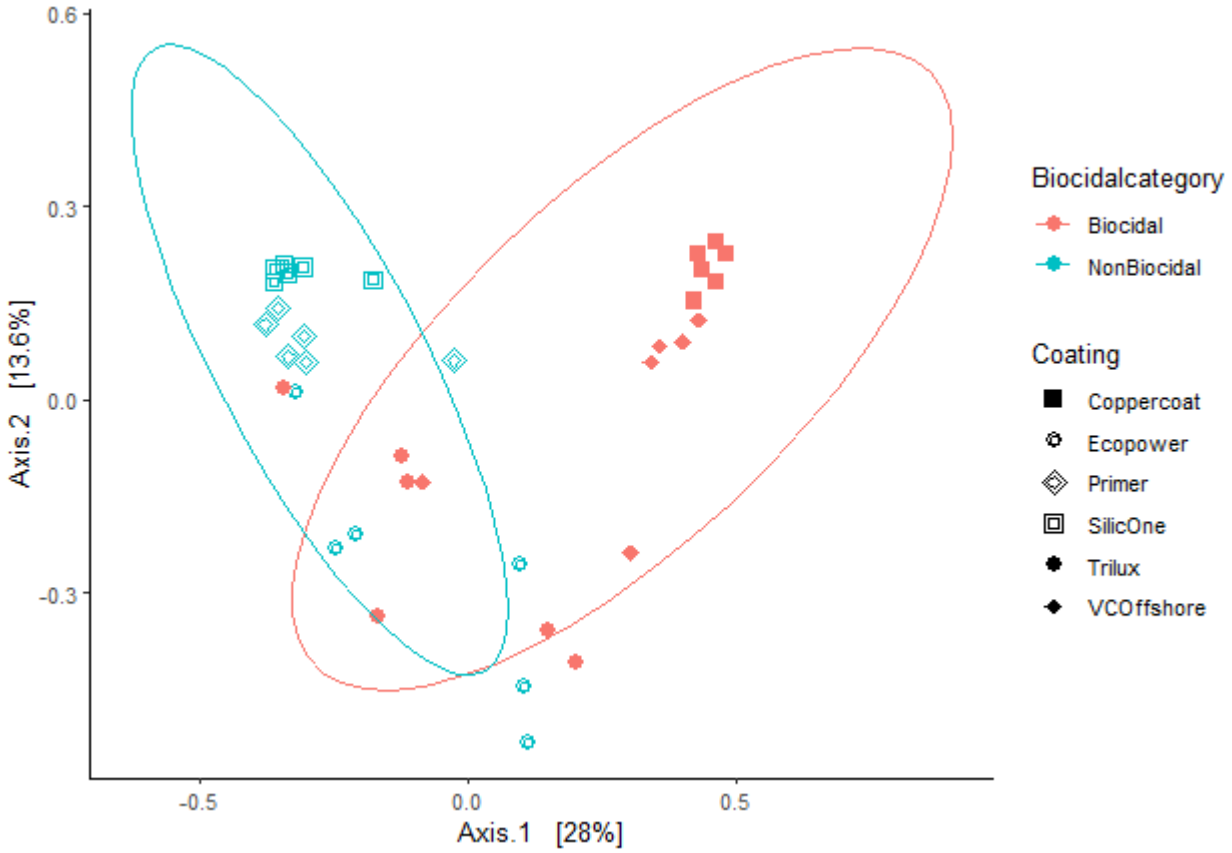


Figure 2.11 Bray-Curtis similarity of mature biofilms (21 days and older) by coating (shape) and biocidal treatment (colour). Ellipticals at 95%.

2.3.5.3 Mature Biofilm Community Composition

There were many shared taxa between treatments, although in varying relative abundances (Figure 2.12). The Coppercoat was heavily composed of *Marinobacter spp.*, while *Cycloclasticus spp.* dominated in others. *Alteromonas* were prominent on biocidal treatments such as Coppercoat, Trilux, and VC Offshore. Trilux, VC Offshore, and Ecopower had a high percentage of *Dasania* and *Sphingorhabdus*.

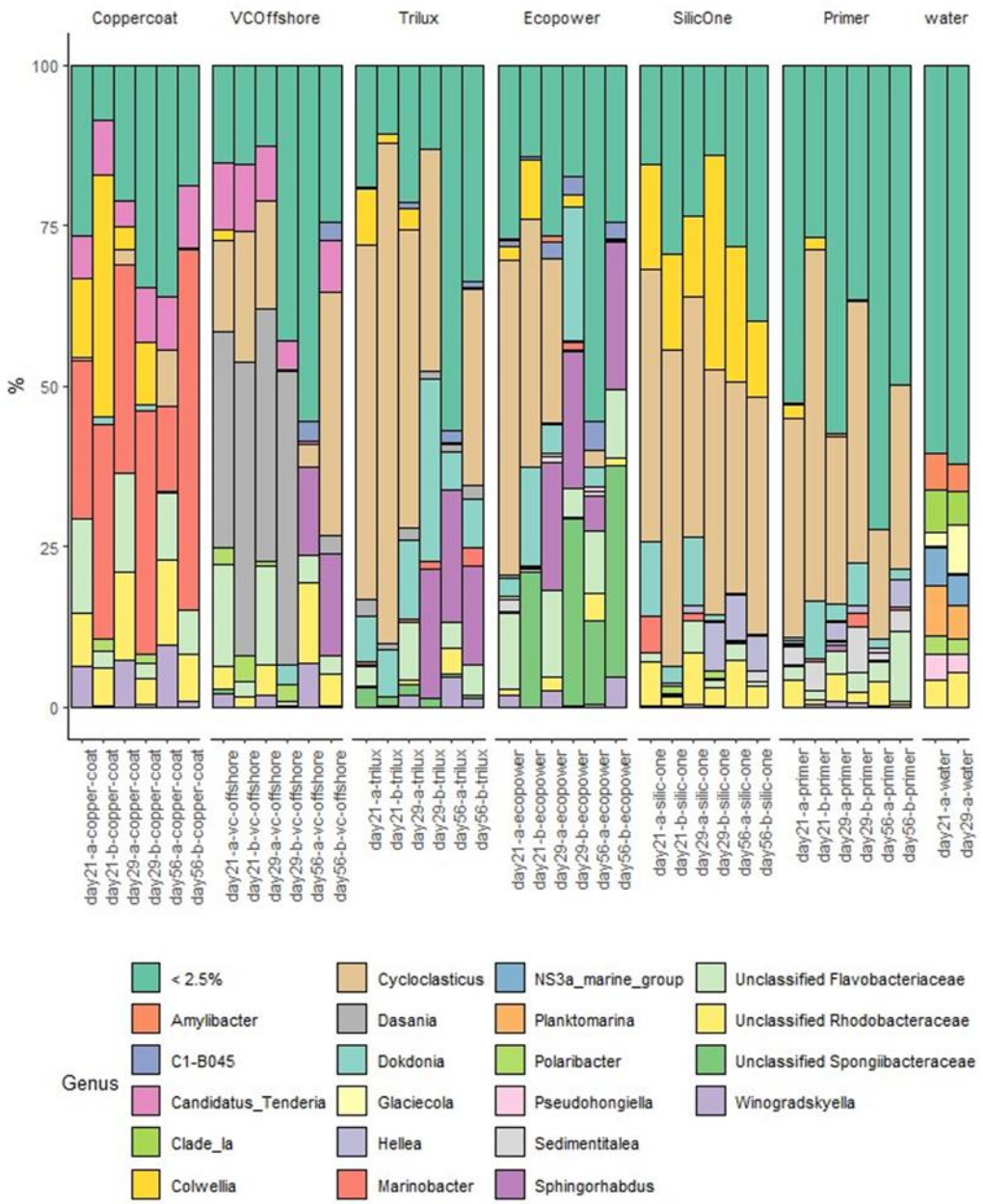


Figure 2.12 Top genus level taxa contributing more than 2.5% to the mature biofilm community by coating.

At the family level (Figure 2.13), the non-biocidal Ecopower biofilm community was similar in composition to that of the VC Offshore and Trilux, albeit with less *Alteromonadaceae*. Coppercoat remains distinct with a large *Marinobacteracea* component. The primer had a significant contribution by *Methylologellaceae*, which was not seen in such proportions in the other treatments. While the Silic One treatment had the largest proportion of *Collwelliacea*.

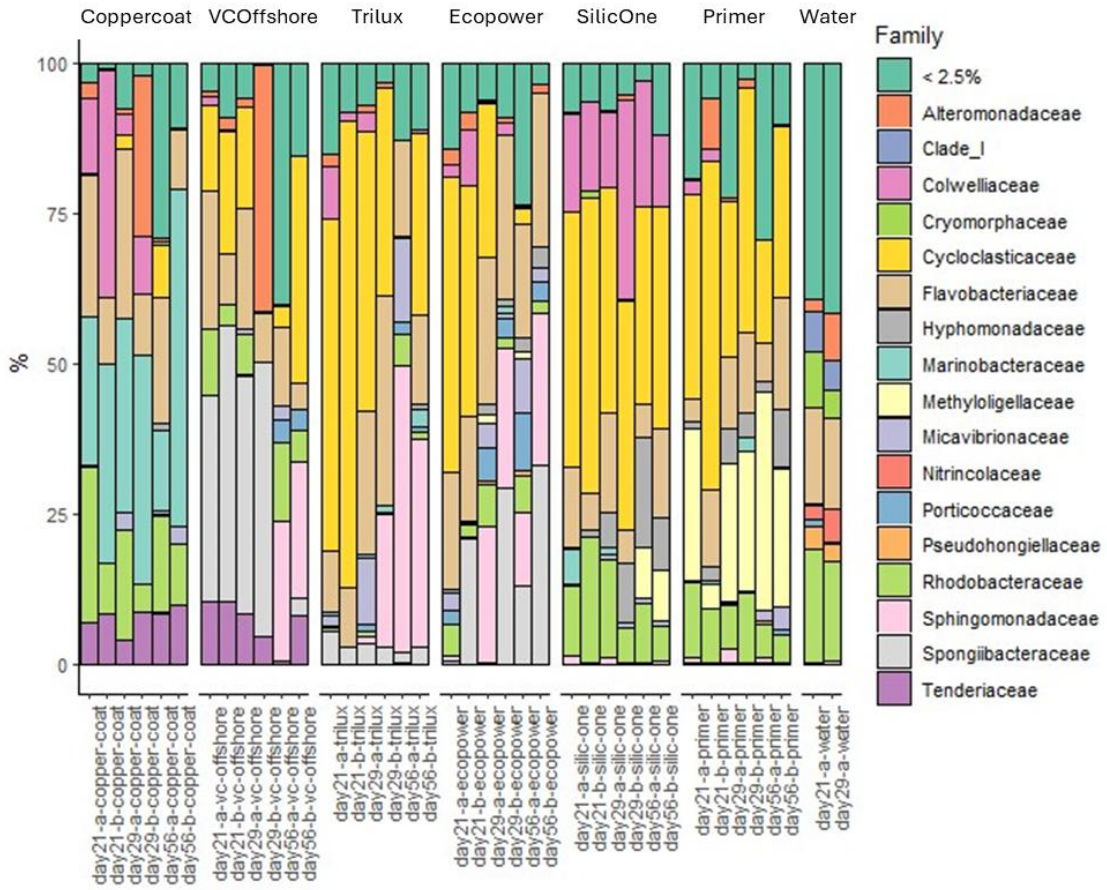


Figure 2.13 Family composition of mature biofilms that contribute more than 2.5% of the community.

2.3.5.4 Mature Biofilm Differential Abundance

DESeq2 analysis showed differential abundance between biocidal and non-biocidal treatments (Figure 2.14). Taxa groups that were found to be differentially abundant in the biocidal treatments include: *Flavobacteriaceae, Spongiibacteraceae, Rhodobacteraceae, Sulfitobacter, Marinobacter, Sphingorhabdus, Colwellia, Wingogradskyella, Methylophaga, Aliikangiella, Crocinitomiceaece, Methylotenera*, unclassified Gammaproteobacteria*, Erythrobacter,* Cellvibrionales*, Mesoflavibacter, Dasania, Nonlabens*, and *Candidatus Tenderia.*

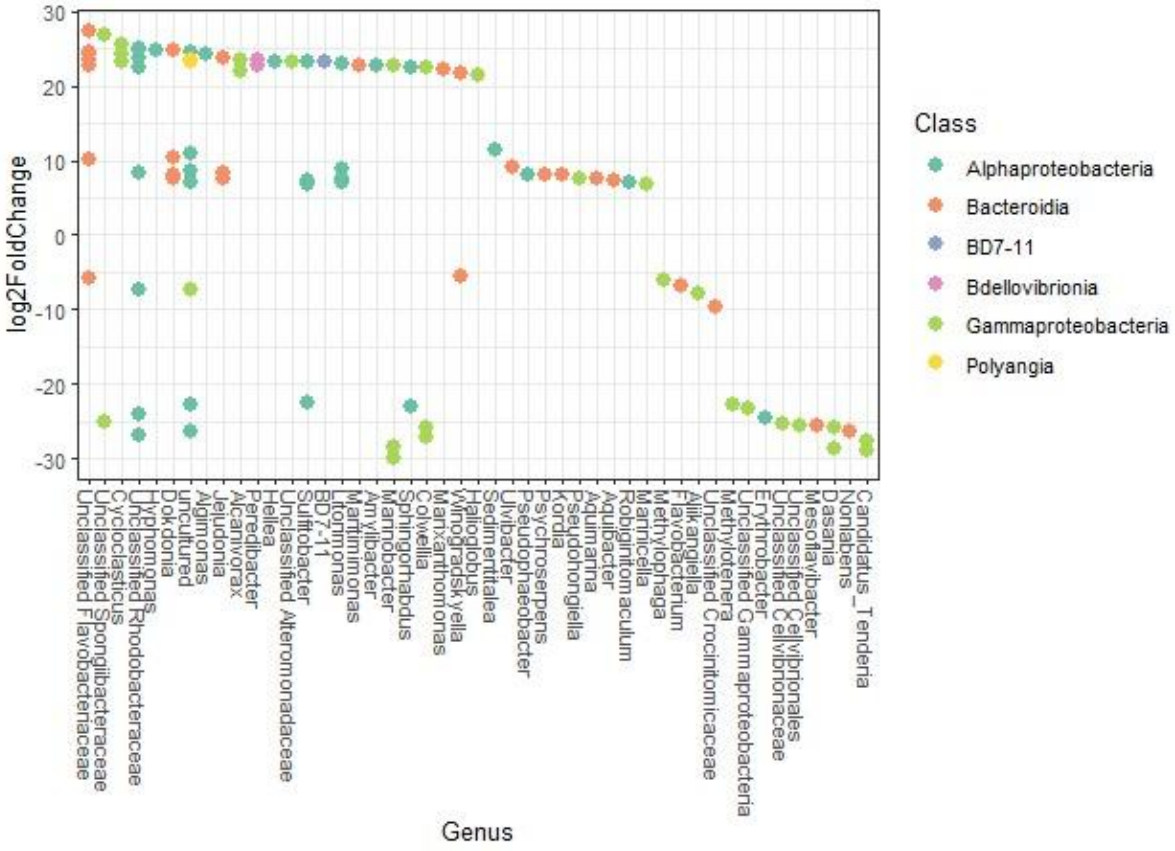


Figure 2.14 DESeq2 analysis by biocidal category: biocidal vs non-biocidal of mature biofilms. Higher log-fold change indicates greater abundance within non-biocidal treatments, and lower logfold change indicates greater abundance within biocidal treatments.

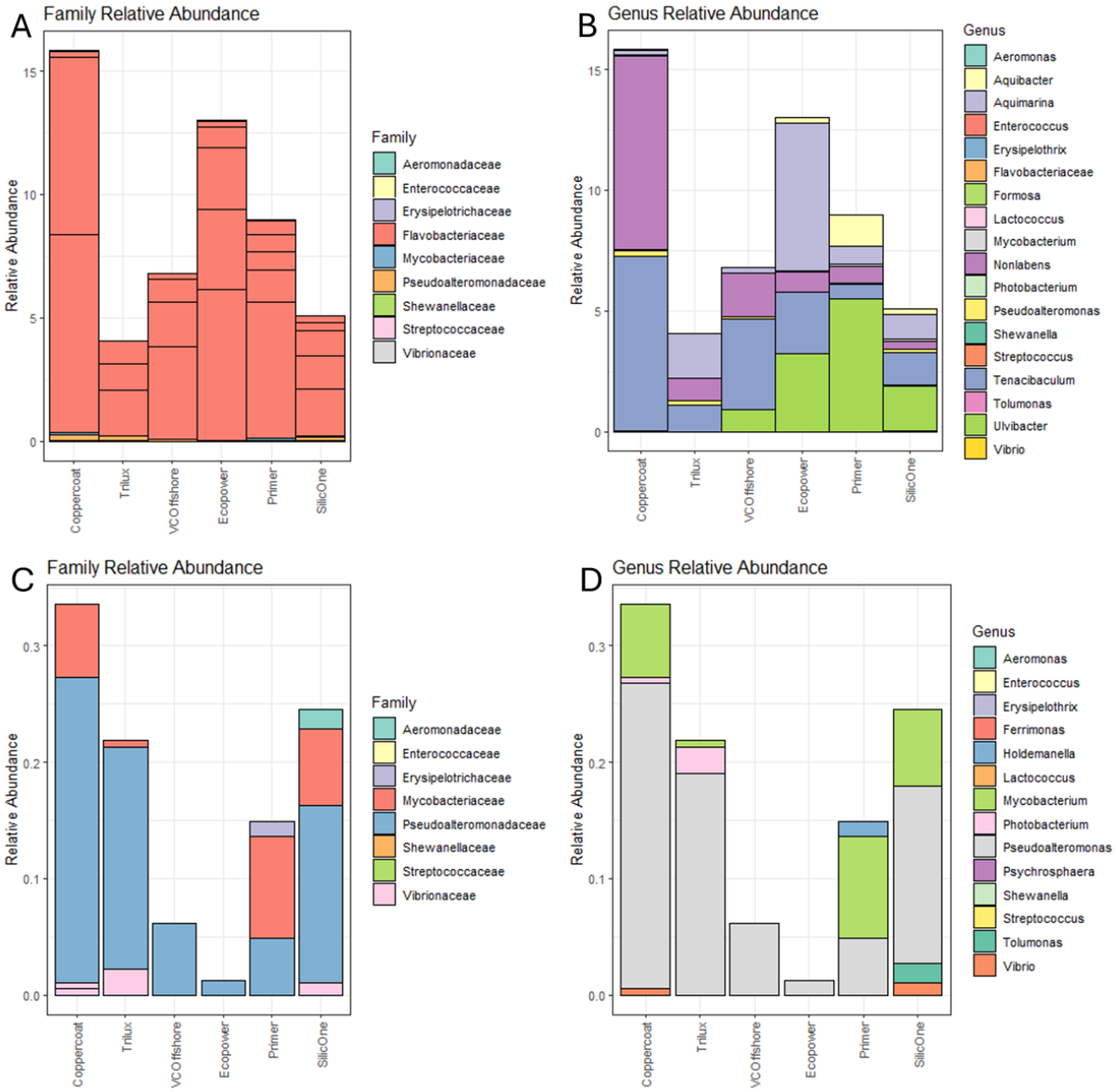
2.3.5.5 Presence of Potential Pathogenic Species

Due to the very low abundance and detection ability of 16S rRNA sequencing, unfiltered reads were used for this investigation. ASVs were filtered for specific taxonomic groups: *Actinobacillus, Aeromonas, Aquibacter, Aquimarina, Campylobacter, Enterococcus, Enterovibrio, Erysipelothrix, Flavobacteriaceae, Formosa, Lacinutrix, Lactococcus, Mycobacterium, Nonlabens, Photobacterium,*

Pseudoalteromonas, Psychroserpens, Shewanella, Streptococcus, Tenacibaculum, Tolumonas, Ulvibacter, and *Vibrio.*

Flavobacteriaceae were very abundant across all six treatments in this study, contributing just over 15% of the community in the Coppercoat biofilms (Figure 2.15A). The biocidal-grown biofilms had relatively large portions of *Nonlabens* sp., *Tenacibaculum* (unidentified species and *T. Dicentrarchi*), *Aquimarina* (unidentified species and *A. longa*), and *Pseudoalteromonas* sp. The VC Offshore biofilms also had a significant contribution from *Ulvibacter* sp., which was also seen in higher abundances in the non-biocidal biofilms (Figure 2.15B)

The biofilms from the non-biocidal coatings were composed of *Aquimarina, Nonlabens* and *Tenacibaculum*, but also had *Aquibacter* sp. present (Figure 2.15B). Only Coppercoat, Trilux (biocidal) and Silic One (foul release non-biocidal) contained members from the *Vibrionaceae* family (Figure 2.15C), and only Coppercoat and the Silic One contained members of the genus *Vibro* (Figure 2.15D). Out of the selected list, Coppercoat, Primer, and SilicOne biofilms showed larger proportions of *Mycobacterium*.



911

912 *Figure 2.15 Relative abundance of potentially pathogenic groups. A and B) potential*
913 *pathogenic taxa heavily dominated by the family Flavobacteriaceae (A). Genus level*
914 *taxa of the flavobacteria groups (B). C and D) potential pathogenic groups with*
915 *flavobacteria removed at family (C) and genus (D) level.*

2.3.6 Microbiome Functional Inference

The overview of KEGG metabolism groups showed a consistency in predicted metabolic function across all coating treatments (Figure 2.16).

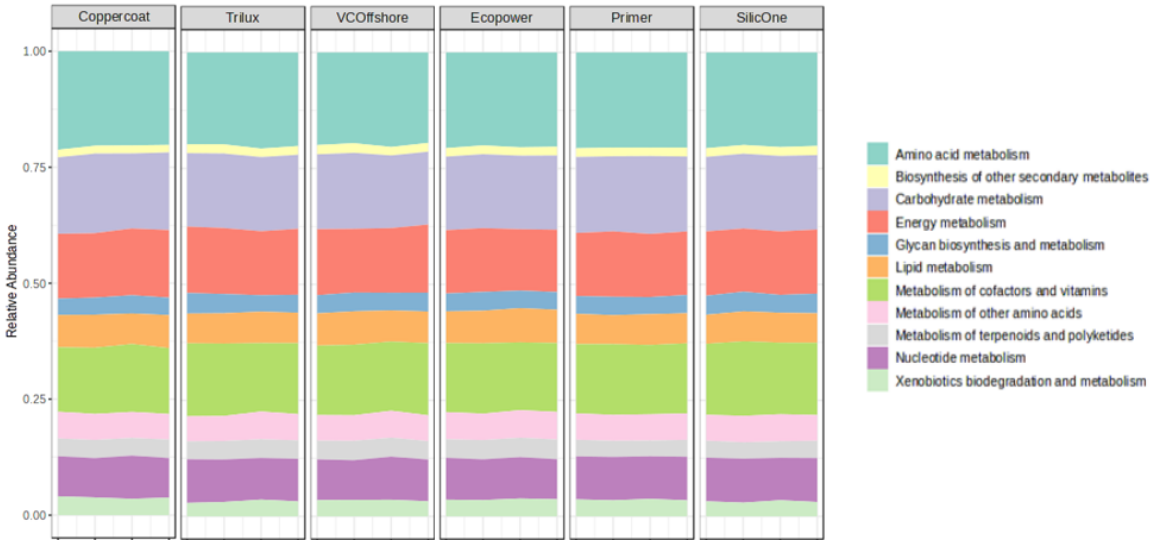


Figure 2.16 Predicted KEGG Metabolism categories of biofilms grouped by coating from days 29 and 56 (mature samples).

Although general metabolic categories were similar, there are some differences in predicted metabolic functions and pathways. Using random forest regression, comparing biocidal categories, there were 13 KEGGs found to be statistically different at 95% confidence between the groups (Figure 2.17, Table 2.2). The KEGGs identified primarily related to metabolism and membrane proteins. K18904 and K18904, which were in the top three predicted KEGGs, relate to an outer membrane protein multidrug efflux system, and were predicted to be abundant in the non-biocidal treatments. When comparisons by coating were conducted (Figure 2.17, Table 2.2), KEGGs related to enzyme activity demonstrated the clearest gradient between biocidal and non-biocidal coatings. 4-hydroxybenzoate-CoA ligase (K04105) predicted abundance was highest in the Silic One biofilm samples then Primer, Ecopower, Coppercoat, VC Offshore and Trilux. 2-dehydro-3-deoxy-L-fuconate 4-dehydrogenase (K18335) predicted abundance was the highest on the Primer coating, then Ecopower, Silic One, VC Offshore, Coppercoat and Trilux. Three of the KEGGs identified in this analysis related to biofilm formation: K12276 “MSHA biogenesis protein MshE PATHWAY: map05111 Biofilm formation - Vibrio cholerae”, K11907 “type VI secretion system protein VasG. map02025 Biofilm formation - Pseudomonas aeruginosa, map03070 Bacterial secretion system”, and K01912 “phenylacetate-CoA ligase. map00360 Phenylalanine metabolism, map05111 Biofilm formation - Vibrio cholerae”. Although there wasn’t a

clear division in biocidal category and their predicted abundance, there were patterns in relation to specific coating treatments. The Coppercoat treatment had the highest abundance for all three KEGGs listed above, and Trilux had the lowest or second lowest abundance. Coppercoat and VC Offshore also had the highest predicted abundance for K11905 “type VI secretion system protein”.

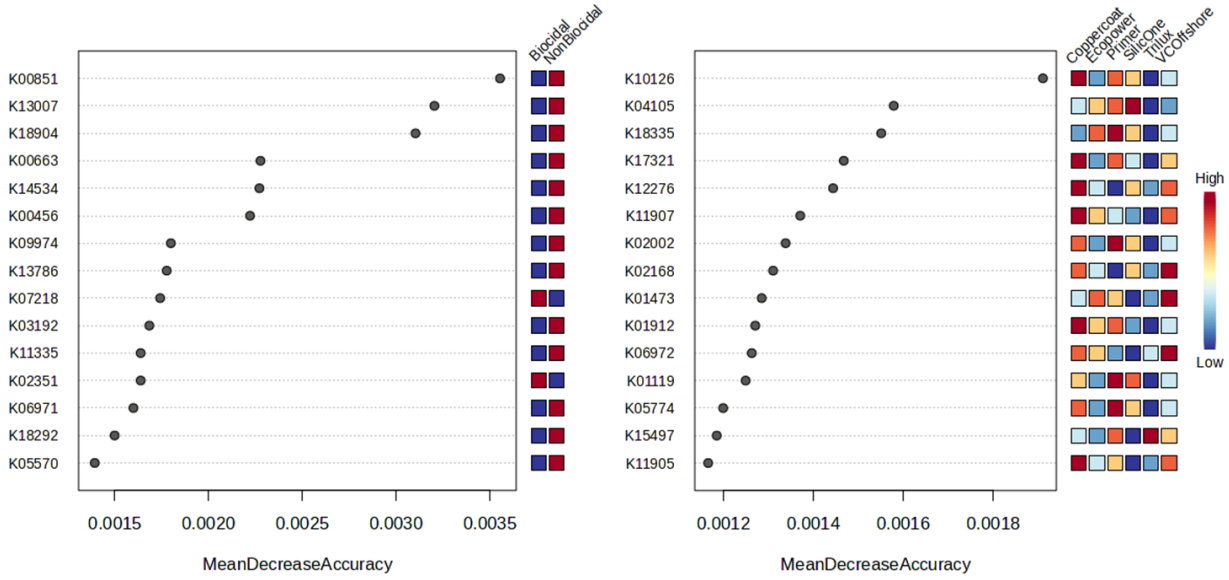


Figure 2.17 Random Forest identification of 13 significant predicted KEGG orthologs. left: analysis between biocidal and non-biocidal treatments, right: analysis between coatings. Red indicates high predicted presence, through a scale to blue which indicates low predicted presence.

Table 2.2 Significant KEGG names and descriptions identified through random forest regression by biocidal category and coating.

KO	**Description**	**By group**
K13007	glycosyltransferase WbpL	Biocidal category
K18904	outer membrane protein, multidrug efflux system nodT, ameC	Biocidal category
K00663	aminoglycoside 6'-N-acetyltransferase aacA	Biocidal category
K14534	4-hydroxybutyryl-CoA dehydratase / vinylacetyl-CoA-Delta-isomeras	Biocidal category
K00456	cysteine dioxygenase CDO1	Biocidal category
K09974	uncharacterized protein	Biocidal category
K13786	cob(ll)yrinic acid a,c-diamide reductase cobR	Biocidal category
K07218	nitrous oxidase accessory protein nosD	Biocidal category
K03192	urease accessory protein ureJ	Biocidal category

K11335	3,8-divinyl chlorophyllide a/chlorophyllide a reductase subunit Z bch	Biocidal category
K02351	putative membrane protein	Biocidal category
K06971	uncharacterized protein	Biocidal category
K18292	(S)-citramalyl-CoA lyase	Biocidal category
K05570	multicomponent Na+:H+ antiporter subunit F mnhF, mrpF	Biocidal category
K10126	two-component system, NtrC family, C4-dicarboxylate transport response regulator DctD	Coating
K04105	4-hydroxybenzoate-CoA ligase	Coating
K18335	2-dehydro-3-deoxy-L-fuconate 4-dehydrogenase	Coating
K17321	glycerol transport system substrate-binding protein	Coating
K12276	MSHA biogenesis protein MshE PATHWAY: map05111 Biofilm formation - Vibrio cholerae	Coating
K11907	type VI secretion system protein VasG. map02025 Biofilm formation - Pseudomonas aeruginosa, map03070 Bacterial secretion system	Coating
K02002	glycine betaine/proline transport system substrate-binding protein	Coating
K02168	choline/glycine/proline betaine transport protein	Coating
K01473	N-methylhydantoinase A	Coating
K01912	phenylacetate-CoA ligase. map00360 Phenylalanine metabolism, map01100 Metabolic pathways, map01120 Microbial metabolism in diverse environments, map05111 Biofilm formation - Vibrio cholerae	Coating
K06972	presequence protease. Disease HO1891 Autosomal recessive spinocerebellar ataxias	Coating
K01119	2',3'-cyclic-nucleotide 2'-phosphodiesterase / 3'-nucleotidase. map00230 Purine metabolism map00240 Pyrimidine metabolism map01100 Metabolic pathways	Coating
K05774	ribose 1,5-bisphosphokinas	Coating
K15497	molybdate/tungstate transport system ATP-binding protein	Coating
K11905	type VI secretion system protein	Coating

2.3.6.1 Investigation of KO relating to Antimicrobial Resistance Mechanisms

Following a post-pipeline filtering step using a pre-defined list of AMR KO orthologs (Batool *et al.*, 2023), out of a potential of 90 AMR KEGGs, 32 were found in the full biofilm dataset, and 30 were found in the 'mature' data subset (Supplementary Table 2.4). Predicted AMR KEGGs are dominated by beta-lactamase classes and aminoglycosides acetyltransferases (Table 2.3).

Table 2.3 List of AMR related Kegg orthologs predicted in all biofilm samples and their descriptions.

KO	**Description**
K00561	ermC, ermA; 23S rRNA (adenine-N6)-dimethyltransferase [EC:2.1.1.184]
K00638	catB; chloramphenicol O-acetyltransferase type B [EC:2.3.1.28]
K03395	aac3-I; aminoglycoside 3-N-acetyltransferase I [EC:2.3.1.60]
K04343	strB; streptomycin 6-kinase [EC:2.7.1.72]
K06979	mph; macrolide phosphotransferase
K08217	mef; MFS transporter, DHA3 family, macrolide efflux protein
K10673	strA; streptomycin 3"-kinase [EC:2.7.1.87]
K17840	aac2-I; aminoglycoside 2'-N-acetyltransferase I [EC:2.3.1.59]
K18220	tetM, tetO; ribosomal protection tetracycline resistance protein
K18231	msrA, vmlR; macrolide transport system ATP-binding/permease protein
K18552	cmlA, cmlB, floR; MFS transporter, DHA1 family, florfenicol/chloramphenicol resistance protein
K18554	cpt; chloramphenicol 3-O phosphotransferase [EC:2.7.1.-]
K18698	blaTEM; beta-lactamase class A TEM [EC:3.5.2.6]
K18781	blaVIM; metallo-beta-lactamase class B VIM [EC:3.5.2.6]
K18790	blaOXA-1; beta-lactamase class D OXA-1 [EC:3.5.2.6]
K18795	blaCARB-1; beta-lactamase class A CARB-1 [EC:3.5.2.6]
K18815	aac6-I; aminoglycoside 6'-N-acetyltransferase I [EC:2.3.1.82]
K18816	aac6-I, aacA7; aminoglycoside 6'-N-acetyltransferase I [EC:2.3.1.82]
K18845	rmt, armA; 16S rRNA (guanine(1405)-N(7))-methyltransferase [EC:2.1.1.179]
K19097	blaVEB; beta-lactamase class A VEB [EC:3.5.2.6]

K19209	blaOXA-42; beta-lactamase class D OXA-42 [EC:3.5.2.6]
K19216	blaIND; metallo-beta-lactamase class B IND [EC:3.5.2.6]
K19217	blaCARB-17; beta-lactamase class A CARB-17 [EC:3.5.2.6]
K19218	blaCARB-5; beta-lactamase class A CARB-5 [EC:3.5.2.6]
K19271	catA; chloramphenicol O-acetyltransferase type A [EC:2.3.1.28]
K19272	aph3-I; aminoglycoside 3'-phosphotransferase I [EC:2.7.1.95]
K19278	aac6-Ib; aminoglycoside 6'-N-acetyltransferase Ib [EC:2.3.1.82]
K19299	aph3-III; aminoglycoside 3'-phosphotransferase III [EC:2.7.1.95]
K19300	aph3-II; aminoglycoside 3'-phosphotransferase II [EC:2.7.1.95]
K19301	aac6-II; aminoglycoside 6'-N-acetyltransferase II [EC:2.3.1.82]
K19319	blaOXA-134; beta-lactamase class D OXA-134 [EC:3.5.2.6]
K19321	blaOXA-214; beta-lactamase class D OXA-214 [EC:3.5.2.6]

2.3.6.2 Predicted Functional Change During Biofilm Development:

Over time there was a general shift from aminoglycoside 6'-N-acetyltransferase I, chloramphenicol O-acetyltransferase type B, beta-lactamase class A CARB-17, MFS transporter, DHA3 family and macrolide efflux protein predictions in early biofilms to chloramphenicol 3-O phosphotransferase, ribosomal protection tetracycline resistance protein, beta-lactamase class A CARB-17, streptomycin 6-kinase and aminoglycoside 3-N-acetyltransferase in the more mature biofilms (Figure 2.18).

Figure 2.18 Heatmap of predicted AMR-related KEGGs from PICRUSt2 analysis over time across all sample categories.

2.3.6.3 KO Functional Predictions by Coating Type

Samples from day 29 and 56 (mature biofilm) were used to compare coatings according to predicted functional traits. AMR-related KEGGs vary in their distribution between coatings (Figure 2.19). Beta-lactamase classes were found to be the most abundant across all the samples.

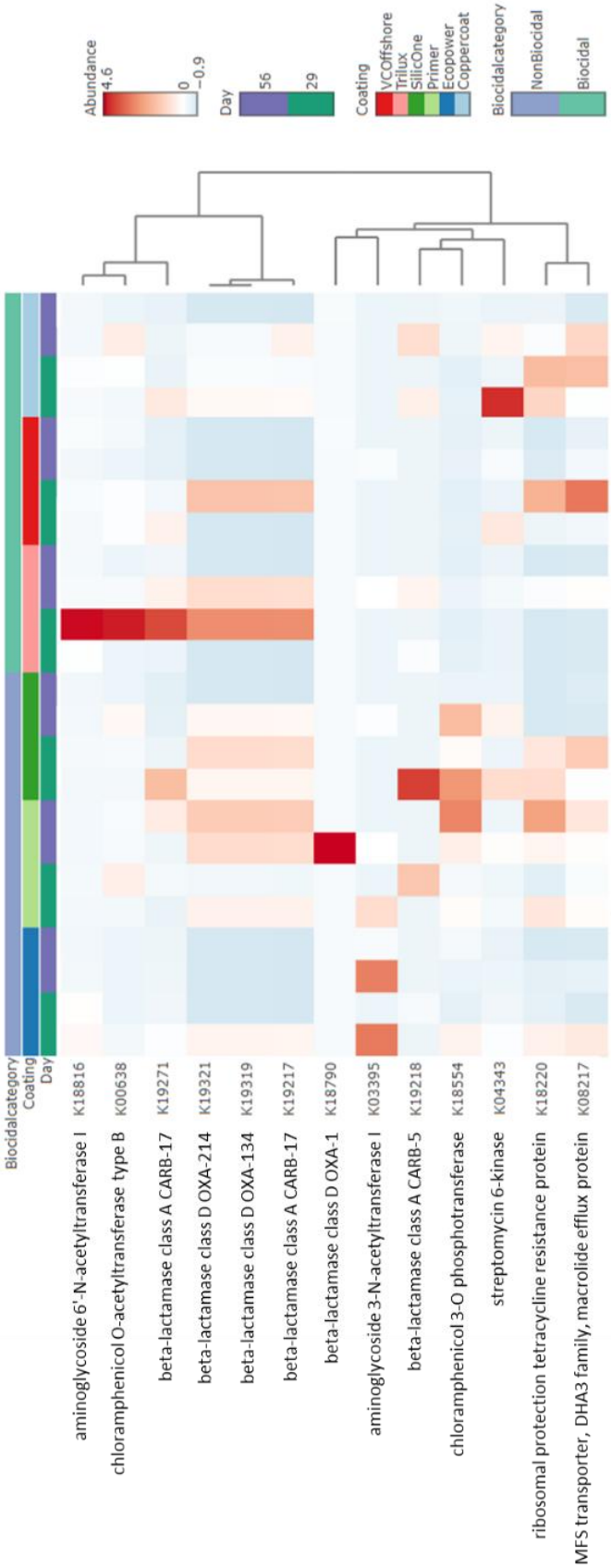


Figure 2.19 Heatmap of predicted AMR KEGGs by coating type for samples days 29-56 only.

Five AMR KEGGs were found to be differentially abundant in the non-biocidal biofilm group of mature samples using White's non-parametric t-test (Figure 2.20). The KEGGs identified relate to two types of beta-lactamase classes (K18781 and K18795), two aminoglycoside acyltransferases (K03395 and K19278) and one chloramphenicol phosphotransferase KEGG (K18854).

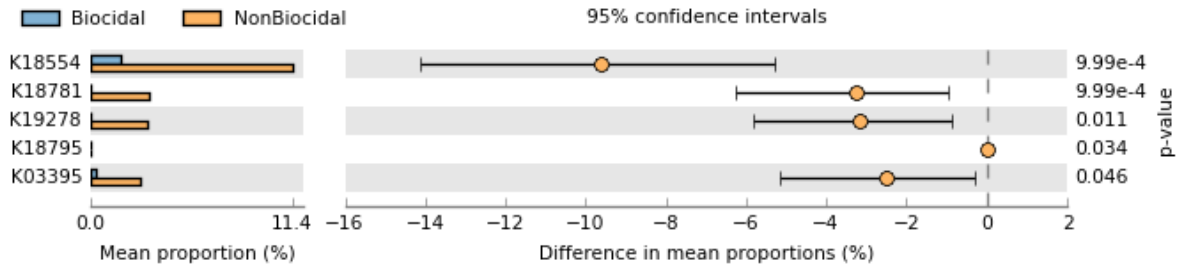


Figure 2.20 White's non-parametric t-test on mature biofilm samples comparing biocidal vs non-biocidal groupings. Yellow coded for non-biocidal group, blue coded for biocidal group. KEGG numbers relate to the following: K18854: cpt; chloramphenicol 3-O phosphotransferase, K18781: blaVIM; metallo-beta-lactamase class B VIM, K18795: blaCARB-1; beta-lactamase class A CARB-1, K19278: aac6-Ib; aminoglycoside 6'-N-acetyltransferase Ib, K03395: aac3-I; aminoglycoside 3-N-acetyltransferase I.

Six AMR KEGGs were found to be statistically different at 95% confidence between specific coatings using Kruskal-Wallis H test (Figure 2.21). K19097 "blaVEB; beta-lactamase class A VEB" was highly predicted in the primer coating biofilms (Figure 2.21A). K19278 "aac6-Ib; aminoglycoside 6'-N-acetyltransferase" and K18781 "blaVIM; metallo-beta-lactamase class B VIM" was highly predicted in the Silic One biofilms (Figure 2.21B and E). The remaining three KEGGs were present across all biofilms, but K18554 was predicted more in the non-biocidal coating treatments (Primer, Silic One, Ecopower) (Figure 2.21F), whereas K0434 was predicted more in the Coppercoat biofilms (Figure 2.21C).

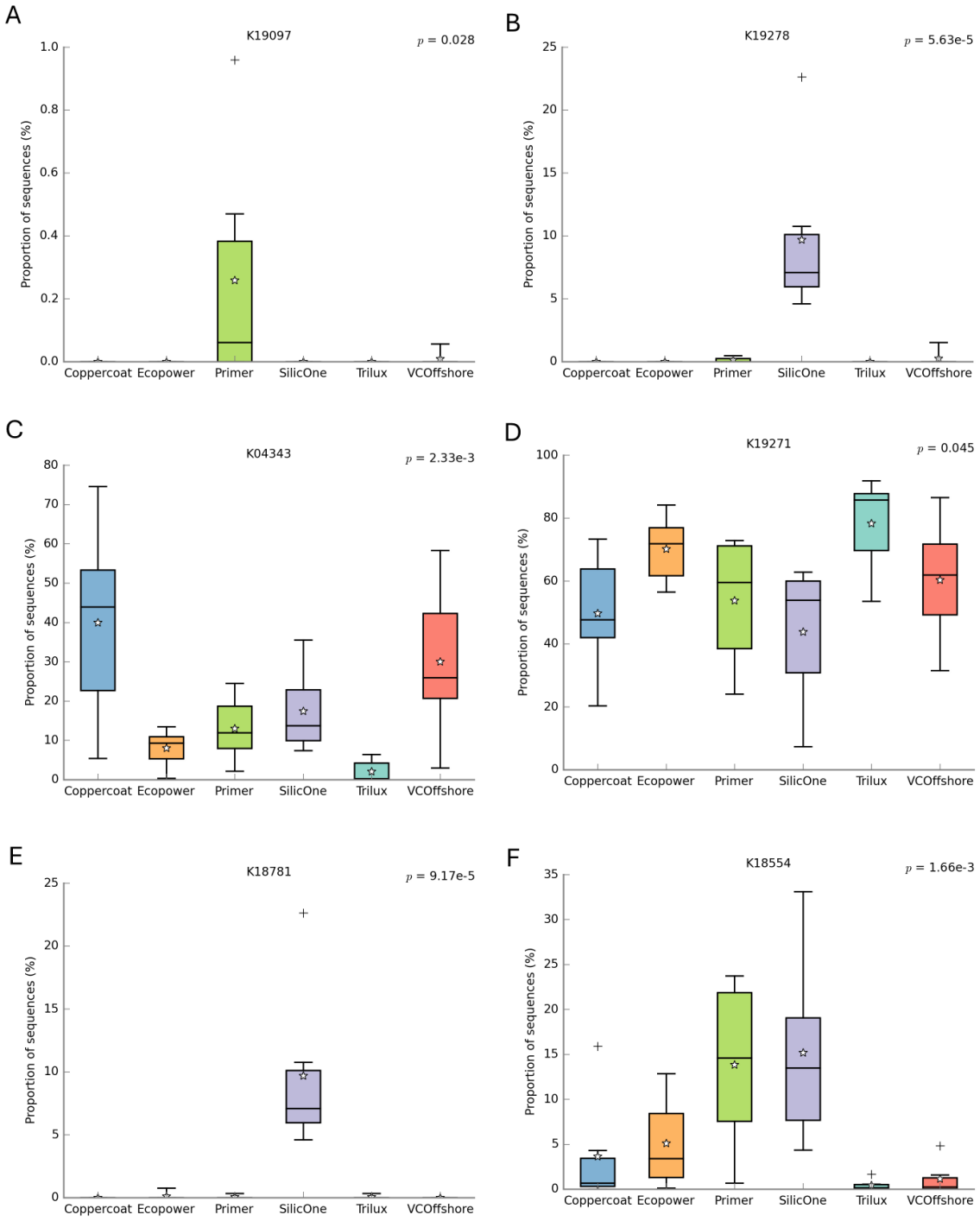


Figure 2.21 (A-F) Kruskal-Wallis H test of AMR KEGGs on mature biofilm samples. (A) K19097: beta-lactamase class A, (B) K19278: acetyl-CoA:kanamycin-B N6'-acetyltransferase, (C) K04343: streptomycin 6-phosphotransferase, (D) K19271: chloramphenicol O-acetyltransferase, (E) K18781: beta-lactam hydrolase (F) K18554: chloramphenicol 3-O phosphotransferase

2.3.7 Metagenome Classification and ARG Detection

Shotgun sequencing was conducted to supplement the 16S community analysis to identify resistance/resistome profiles, which is not achievable with 16S data. Community composition using shotgun sequencing, generated through Kraken and Bracken pipelines were heavily dominated by Gammaproteobacteria, followed by Alphaproteobacteria and Flavobacteriia at the class level (Figure 2.22). At the genus level, *Cycloclasticus* was the most prevalent across all samples, apart from the Coppercoat biofilm, which was dominated by *Marinobacter* (Figure 2.23, Supplementary Table 2.5, Supplementary Figure 2.4-7).

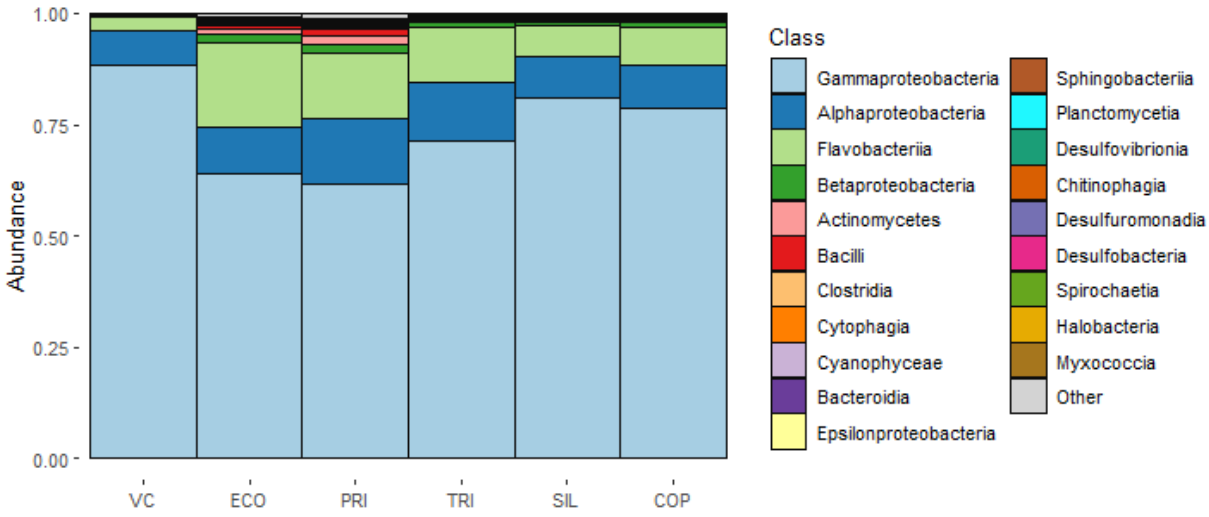


Figure 2.22 Kraken and Bracken results of shotgun sequencing of mature biofilms from each coating at the class taxonomic level. VC: VC Offshore. ECO: Ecopower. PRI: Primer. TRI: Trilux, SIL: Silic One. COP: Coppercoat

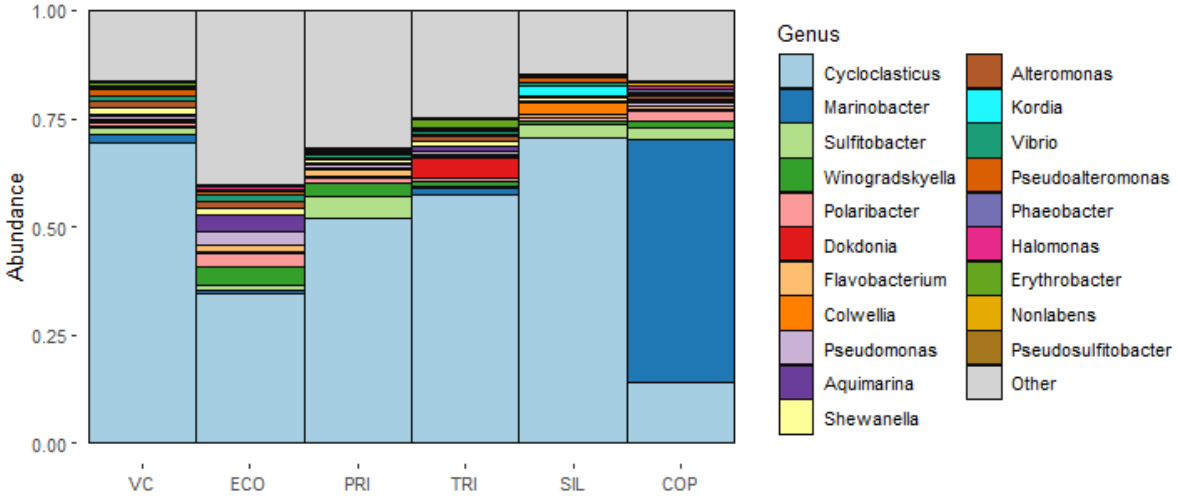


Figure 2.23 Kraken and Bracken results of shotgun sequencing of mature biofilms from each coating at Genus taxonomic level. VC: VC Offshore. ECO: Ecopower. PRI: Primer. TRI: Trilux, SIL: SilicOne. COP: Coppercoat

An assembly was generated using the assembler metaSPAdes, with the subsequent contigs processed with ABRicate against MEGARES and VFDB databases (Supplementary Table 2.6). Only Coppercoat biofilms provided positive hits for AMR genes above 80% similarity (default settings). Genes for aminoglycoside resistance (APh3-DPRIME, APH6), trimethoprim (DFRA6) and sulfonamide resistance (SUL1) were found in the Coppercoat biofilm sample. All biofilm samples had positive hits for virulence factors, specifically QACK, acpXL, mbtH-like, pilG, pilT and pilU (Figure 2.24).

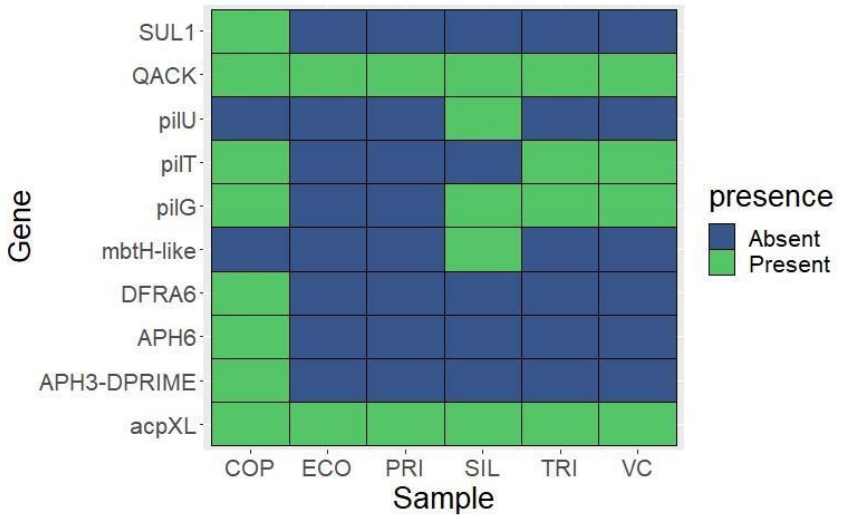


Figure 2.24 Presence-absence heatmap of ABRicate results from the 6 coating types: VC: VC Offshore. ECO: Ecopower. PRI: Primer. TRI: Trilux, SIL: SilicOne. COP: Coppercoat.

However, when using the ARGs-OAP pipeline, multiple ARG groups were detected within all biofilm samples, which were heavily dominated by polymyxin-resistant classes and multidrug-resistant classes. Only the Coppercoat biofilm samples had high levels of aminoglycoside-related genes detected (Figure 2.25).

When the multidrug-resistance subtypes were assessed, it was evident that the biocidal treatments were relatively similar in their subtype composition. Interestingly, the non-biocidal Ecopower treatment appeared more like the biocidal category compared to other non-biocidal coatings (Figure 2.26). The polymyxin class was made up of the polymyxin_ugd subtype exclusively across all samples (Figure 2.27). However, the Ecopower had only one type of beta-lactam gene present, compared to a high diversity of beta-lactam-resistant classes in the biocidal groups (Supplementary Figure 2.10)

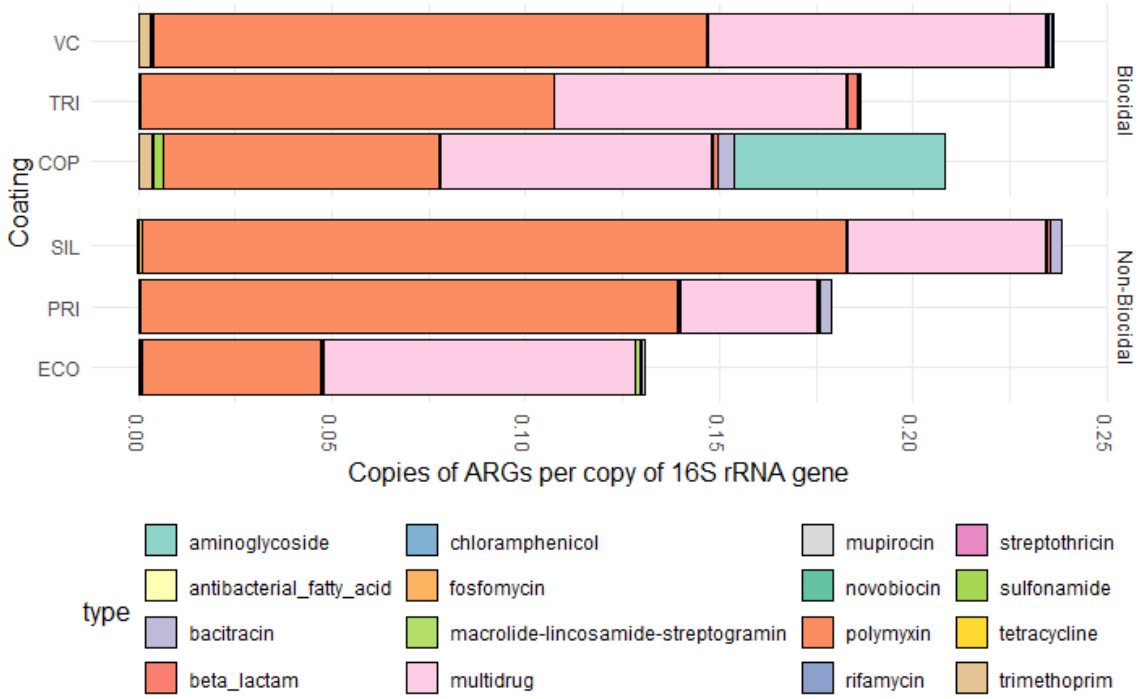


Figure 2.25 ARGs-OAP pipeline of ARG-types normalised against 16S gene copies for each treatment.

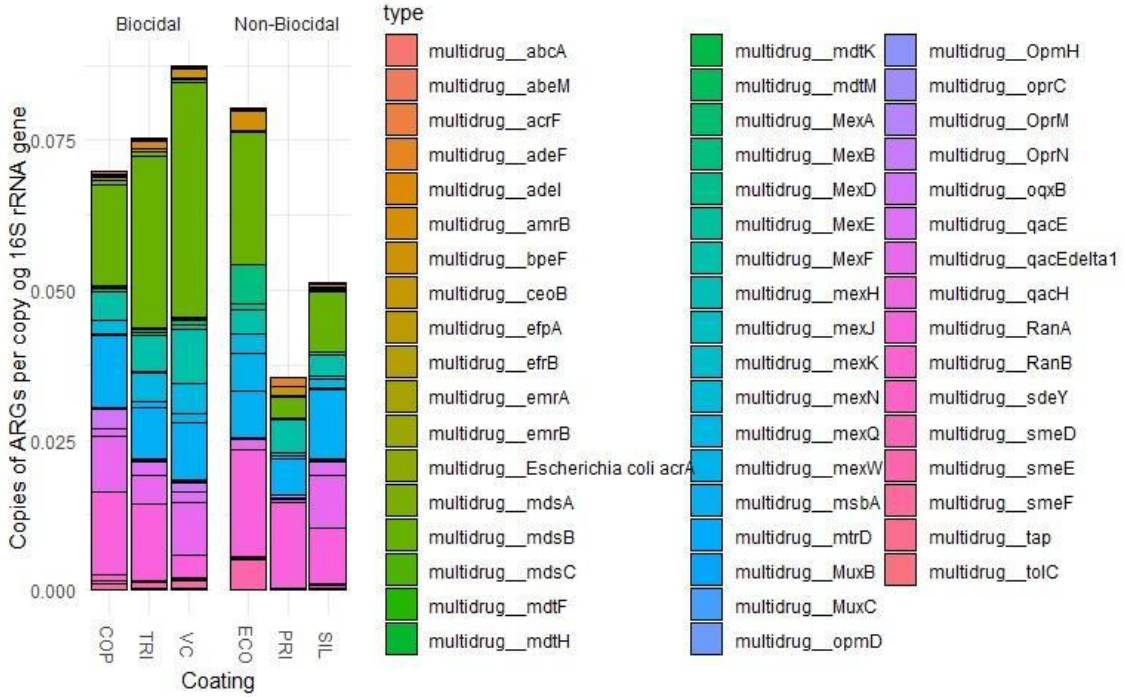


Figure 2.26 Breakdown of the subtypes of multidrug-resistant classes found in each treatment (normalised against 16S gene copies).

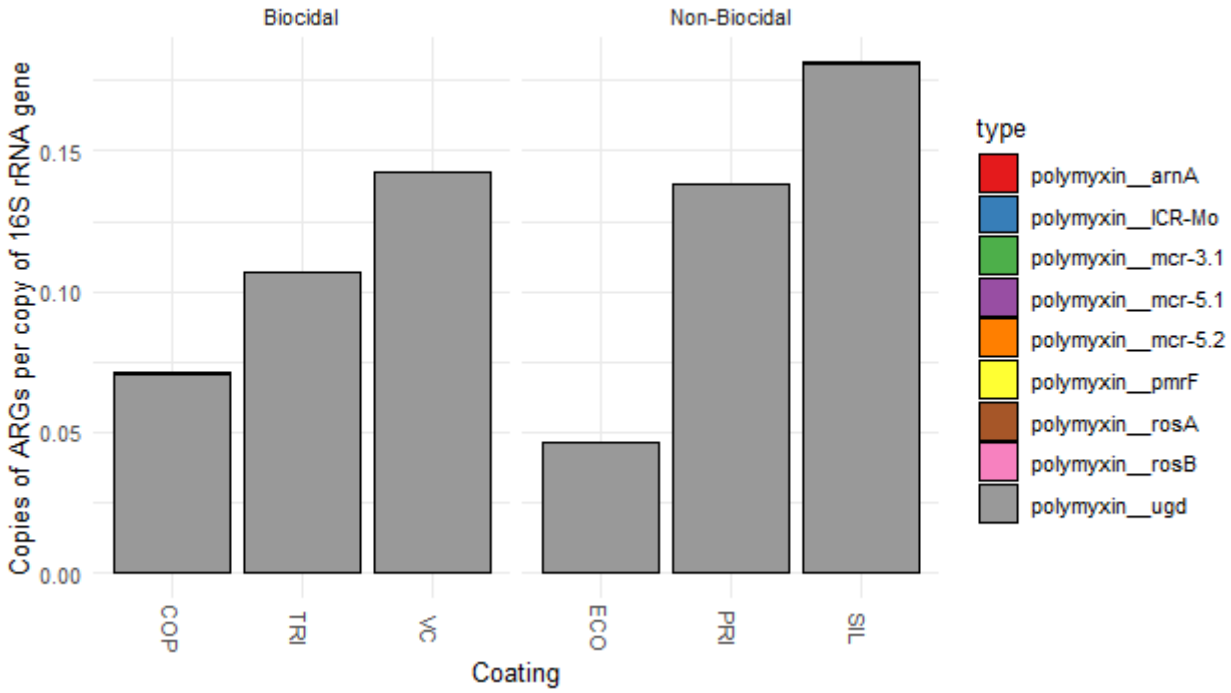


Figure 2.27 Breakdown of the subtypes of polymyxin-resistant classes found in each treatment (normalised against 16S gene copies)

2.3.7.1 Metagenome Resistome Risk Scores

Resistome risk was estimated using MetaCompare 2.0, (Figure 2.28). Human Health resistome risk (HHRR) was low, with risk scores from zero (Ecopower idba and MEGAHIT assemblies) to 0.029 (VC Offshore MEGAHIT assembly). Ecological resistome risk (ERR) was comparatively higher, with risk factors between 1.83 (Coppercoat MetaSPAdes assembly) and 6.08 (Trilux MEGAHIT assembly). The assembly method used, on average, gave similar results, with the exception of biocidal ERR scores. There is a significant difference between biocidal and non-biocidal HHRR scores (Wilcoxon p=0.0081) based on all three assembly approaches.

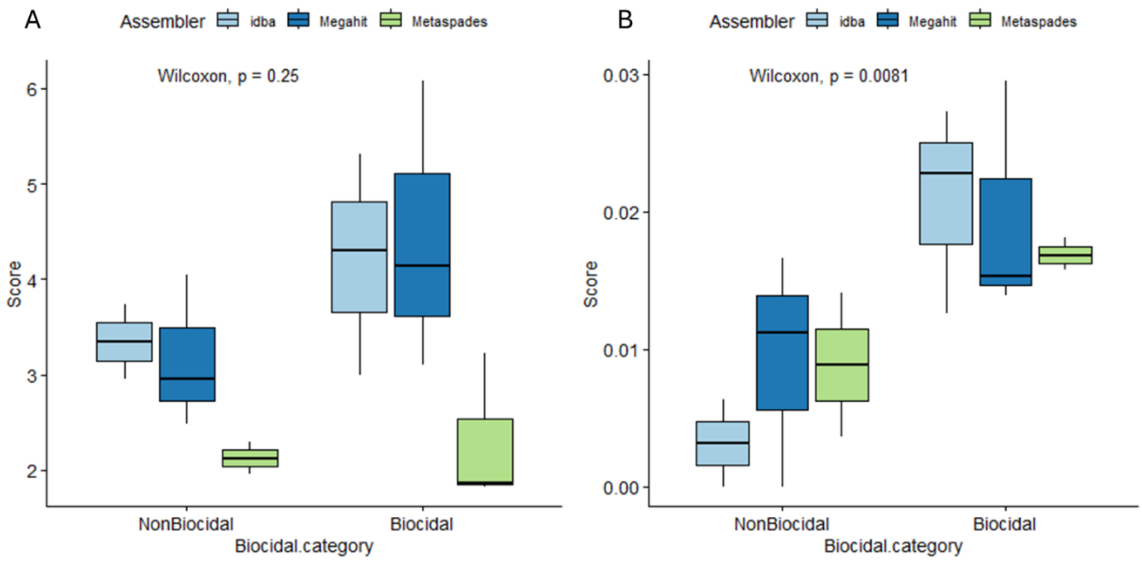


Figure 2.28 MetaCompare 2.0 resistome risk scores by biocidal category and assembly technique. A) Ecological resistome risk (ERR) from 2 to 6. Wilcoxon between biocidal category: W=43, p=0.25 B) Human health resistome risk (HHRR) from 0 to 0.03. Wilcoxon between biocidal category: W=57, p=0.008

2.4 Discussion

In this study, bacterial microbiome and metagenomic analyses were used to characterize and define the biofilm microbial communities associated with various anti-fouling biocidal and non-biocidal coatings. Although biofilms from different treatments/coatings shared similar dominant taxa, there were treatment-unique ASVs, producing unique community profiles e.g. unique ASVs made up 2.6% of the biofilm on Coppercoat coating (Supplementary Figure 2.2). The presence of potentially pathogenic genera were detected at low abundances and were coating treatment-specific. Using metagenomic shotgun sequencing, antibiotic resistance gene profiles were created, highlighting strong potential for polymyxin and multi-drug resistance across all treatments, as well as aminoglycoside resistance in the Coppercoat biofilm samples. Resistome risk scores were also created based on resistance gene and community profiles, highlighting increased 'risk' in the biocidal treatments compared to the non-biocidal treatments.

2.4.1 Coating-Specific Community Composition Changes

In general, the diversity of all biofilms rapidly dropped after initial colonisation (Figure 2.7), while also forming distinct communities compared to the surrounding seawater

(Figure 2.9). The dominant taxa of these early colonisers were similar across the coatings although in varying quantities (*Alteromonas, Oceaniserpentilla, Oleispira* and *Pseudoalteromonas)*, except for the biocidal Trilux and VC Offshore, which showed a higher variability and lower relative abundance in its dominant taxa. This is likely due to the inhibitory properties of the biocidal coatings, causing reduced diversity and inhibitory effects on the settling taxa.

The most common genus, *Alteromonas,* are Gram-negative obligate aerobic heterotrophs that are found in a diverse array of marine environments. They typically display extensive degradative properties and can grow with minimal nutritional requirements (López-Pérez and Rodriguez-Valera, 2014), making them excellent competitors in biofilm colonisation. *Oceaniserpentilla haliotis* is widely found on marine organisms, in seawater and sediment (Schlösser *et al.*, 2008), and exhibits characteristics linked to the degradation of petroleum compounds and secretion of bactericidal compounds (Hazen *et al.*, 2010; Satomi and Fujii, 2014). *Oleispira* is a genus of marine obligate hydrocarbon-clastic bacteria (OHCB) (Timmis, 2010). They are recognised as potentially important organisms capable of oil degradation in the marine environment. Physical–chemical properties of hydrocarbon compounds influence their behaviour, especially at low temperatures (Golyshin *et al.*, 2010). These genus groups have a general common characteristic: their ability to break down hydrocarbon compounds. This ability may complement their dominance as early colonisers on the artificial coatings of this study, as well as the location of being in a working and polluted port.

Pseudoalteromonas is the most abundant bacterial genus found in coastal waters (Sun *et al.*, 2023), therefore, its abundance in this study wasn't surprising. It comprises of chemoheterotrophic gram-negative bacteria that thrive aerobically. *Pseudoalteromonas* have been well documented for their production of low and high-molecular weight compounds linked to: antimicrobial, anti-fouling, algicidal and antibiotic properties (Holmstram and Kjelleberg, 2006; Shnit-Orland, Sivan and Kushmaro, 2012; Offret *et al.*, 2016; Chau *et al.*, 2021). Species of *Alteromonas* and *Pseudoalteromonas* are generally seen as early-coloniser species (Grasland *et al.*, 2003; Dang and Lovell, 2016), which correlates with their presence in this study and the early biofilms. Like many other biofilm species, these bacteria can produce extracellular polymeric substances (EPSs), which form the functional and structural integrity of marine biofilms. This thin layer of organic matter traps nutrients from the

water column, which may provide protection to other bacteria by blocking the transfer of toxic anti-fouling compounds (Costerton *et al.*, 1995; Chen *et al.*, 2013).

As the biofilm developed, there was a clear community compositional shift and change in the dominant taxa (Figure 2.8, Figure 2.9, Figure 2.12, Supplementary Figure 2.12). Although the composition of early colonisers varied by coating type, and consisted of highly diverse communities, they typically consisted of *Flavobacteria, Pseudoalteromonas, Oleispira, Oceaniserpentilla, Fluviicola*, and *Halioglobus*. By day 7, community compositions became dominated by *Aestuariicella, Alteromonas, Cycloclasticus, Colwellia* and *Dasania*. By day 21, there was another community composition shift, which was more diverse than the mid-aged biofilms, exhibiting coating-specific compositions. Dominant taxa from all coatings included: *Candidatus_Tenderia, Colwellia, Marinobacter, Flavobacteriaceae, Aestuariicella, Cycloclasticus, Dasania, Rhodobacteraceae, Winogradskyella,* and *Sphingorhabdus*. Similarly, Chen *et al.* (2013) found the largest community shift in biofilms on copper coatings occurred between week 1 and 2 from *Alteromonas*-dominated biofilms to *Rhodobacteraceae*, *Erythrobacter* and *Cycloclasticus* dominated biofilms in week 2. Chen et al. (2013) also identified another minor community shift at week 4, similar to what was seen in this study from 21 days onwards (Chen *et al.*, 2013).

In general, the biocidal coatings had lower diversity metrics than the non-biocidal treatments, indicating a biocidal selective effect (Figure 2.7, Figure 2.10). *Alteromonas*, which dominated the early biofilms on all coatings, is seen at lower relative abundances on the non-biocidal coatings while remaining one of the dominant taxa in the biocidal communities (Figure 2.12, Figure 2.14). *Oceaniserpentilla, Oleispira* and *Pseudoalteromonas*, which were key players in the early biofilm, were all absent from the top 20 taxa across all coating types in the mature biofilm communities (Figure 2.12).

One of the key core taxa that was found across all coating types, is *Cycloclasticus,* which was found across all mature biofilm samples in this study (Figure 2.12). It is a Gram-negative Gammaproteobacteria and is widely distributed in marine sediments, both in contaminated coastal areas and in deep-sea mid-oceanic sediments (Dong *et al.*, 2015; Vincent, Jennerjahn and Ramasamy, 2021). The genus name, *Cycloclasticus*, refers to its remarkable ability to degrade the polycyclic aromatic hydrocarbon (PAH) rings such as naphthalene, phenanthrene, and pyrene, forming

simpler products. Its ability to break down PAHs contributes to the surrounding ecosystem's health and balance (Staley, 2010), and potentially its ability to thrive on the anti-fouling coatings tested in this study due to the large composition of aromatic hydrocarbons within the coatings themselves (Supplementary Table 2.7). There was also likely a large abundance of PAHs within the study environment based in the Port of Blyth (Woodhead, Law and Matthiessen, 1999). *Cycloclasticus* was seen in smaller relative abundances within the Coppercoat biofilms, which unlike the other coatings tested in this study, was an epoxy-based coating, and therefore doesn't contain PAHs within the coating itself. In comparison, however, Silic One did contain higher relative abundances of *Cycloclasticus* and is a silicone-based coating. Although the coating doesn't contain PAHs itself, silicone has the ability to absorb PAHs from the environment (Connell *et al.*, 2014; Hamzai *et al.*, 2021).

The biofilm community within the Coppercoat treatment, formed the most distinct community when assessing diversity metrics and community composition profiles (Figure 2.11, Figure 2.12). The dominant taxa included: *Alteromonas, Candidastus Tendaria, Colwellia, Cycloclasticus, Winogradskyell* and *Marinobacter*. The latter, which is a highly efficient degrader of aliphatic and polycyclic hydrocarbons (Grimaud, 2010; Liu *et al.*, 2021; Peña-Montenegro *et al.*, 2023), dominated the Coppercoat biofilm community and was uniquely characteristic compared to the other coatings (Figure 2.12). Similarly, Chen *et al.* (2013), who tested copper-based coatings in Singapore, found that biofilms were also dominated by *Alteromonas sp., Marinobacter sp.* and *Cycloclasticus sp.* Interestingly, *Marinobacter* and *Candidatus Tenderia* possess electrochemical properties, and their presence in this study coincides with the coatings that contain copper filings (Coppercoat and VC Offshore). Specifically, *Candidatus Tenderia electrophaga*, (found in this study at 92-95% confidence and the only species of the genus) is an uncultivated electro-autotroph that has previously been identified from biocathode enrichment (Eddie *et al.*, 2016). It is electroautotrophic, meaning It utilizes electrons from a cathode to fix CO_2 (Eddie *et al.*, 2016). The copper fillings in the paint and the interaction with the surrounding marine environment could be causing cathode-like interactions between the coatings and the colonising biofilm community. However, further study and analysis would be needed to confirm this.

Trilux and Ecopower biofilms appeared similar to each other when comparing dominant taxa (not taking into consideration taxa that were present at less than 2.5% relative abundance). Taxa included *Aestuariicella, Cycloclasticus, Dasania, Dokdonia*

and *Sphingorhabdus*. *Aestuariicella* has one known species, *A. hydrocarbonica*, which was first isolated from oil spill-contaminated tidal flat sediments in Dangjin Bay, South Korea (Lo *et al.*, 2015). This species has a strong association with plastic debris and is capable of degrading hydrocarbon polymers, although it does not degrade plant polymers like starch (Lo *et al.*, 2015). It is sensitive to tetracycline but resistant to other common antibiotics. While *A. hydrocarbonica* is rare in seawater, it has been found in various locations, suggesting a potentially broad geographic distribution or association with areas of high human impact (Lo *et al.*, 2015), which correlates with its presence on marine paints within a busy port. *Dasania,* which consists of two species (*D. marina* and *D. phycosphaerae*) are relatively understudied in regards to its functions (Lee *et al.*, 2007; Jiang *et al.*, 2023). *Dokdonia* belongs to the family *Flavobacteriaceae* and has been demonstrated to form synergistic-resistant biofilms (Synergistic-resistant biofilms are bacterial communities that are resistant to antimicrobial agents and bacterial invasion due to synergistic interactions between multiple species) (Burmølle *et al.*, 2006; Bandini *et al.*, 2021). It can have mixed phototroph and chemotroph abilities, with research focusing on its potential contributions to the marine food web and dynamics (González *et al.*, 2011). *Sphingorhabdus* spp*.* are commonly isolated from a range of habitats (soils, freshwater, marine habitats, and activated sludge) and can play roles in the degradation of xenobiotic and recalcitrant aromatic compounds, which can be of natural or anthropogenic origin (Jeong, Jin and Jeon, 2016). This makes them significant for environmental bioremediation processes (Yang, Lee and Cho, 2023).

Colwellia, which was found in varying relative abundances across the coating types, but primarily in the Coppercoat and Silic One coatings, is a genus of bacteria known for its adaptation to extreme marine environments (Peoples *et al.*, 2020). These bacteria have adaptations, such as variations in membrane fluidity and respiration, which are critical for coping with high pressure and low temperatures in the deep ocean (Peoples *et al.*, 2020). These characteristics may help *Colwellia* sp. succeed on the ’undesirable’ anti-fouling coatings, both biocidal and nonbiocidal (foul release).

In this study, taxa that were identified as being highly differentially abundant in the biocidal treatments include *Sulifitobacter, Winogradskyella, Erythrob*acter and *Mesoflavibacter* (Figure 2.14)*.* As these taxa can be indicative of polluted human-impacted environments (Kalaitzidou *et al.*, 2022), it is unsurprising that they were detected on the biocidal coatings in the Port of Blyth due to shipping and industrial

activity (Woodhead, Law and Matthiessen, 1999). While these communities are dominated by similar taxa (Figure 2.5, Figure 2.13), there are large proportions of these communities that are made up of 'rarer taxa' that make up less than 1% of the community. This is where the unique taxa from each coating will be found (Supplementary Figure 2.2). For example, the grouped non-biocidal coatings had 39.66% unique ASVs, while the biocidal coatings had 12.14% unique ASVs (Figure 2.5). The Primer control coating had 25.12% unique ASVs, while the remaining coatings all had between 8.1-8.6% unique ASVs, except for the Trilux biofilms which only had 2.34% unique taxa.

Interestingly, consistent with the findings of this study, Flach et al., (2017) and von Ammon et al., (2018) both found that Gammaproteobacteria dominated on their anti-fouling coatings, followed by Alphaproteobacteria and Bacteroidia (Supplementary Figure 2.11). Gammaproteobacteria have been reported to be particularly tolerant to copper-based coatings and associated with stressed environments. The community composition on these anti-fouling coatings have been linked to metal- and/or biocide-tolerant taxa (von Ammon *et al.*, 2018; Catao *et al.*, 2021; Papadatou *et al.*, 2021).
The community composition of all samples in this study looks compositionally different between shotgun data and 16S rRNA data (Figure 2.12 vs Figure 2.23). At the Class level, shotgun analysis using Kraken and Bracken detected Flavobacteriia as the 3rd dominant class after Alphaproteobacteria and Gammoproteobacteria (Figure 2.22), whereas using 16S data, Flavobacteriia was not one of the dominant taxa (Supplementary Figure 2.11). This discrepancy in community composition between the two techniques is likely due to PCR bias in 16S rRNA sequencing and the size of different species genomes in shotgun techniques (Durazzi *et al.*, 2021; Stothart, Mcloughlin and Poissant, 2023; Bars-cortina *et al.*, 2024).

2.4.2 Potential Pathogen Detection

The majority of the pathogens detected using shotgun data belong to the *Flavobacteriaceae* family, which is a large and diverse taxonomic group containing a large portion of animal and human pathogens (McBride, 2014). *Flavobacteriaceae* are abundant across all six treatments in this study, contributing just over 15% of the community in the Coppercoat treatment (Figure 2.15).

The biocidal coatings have relatively large proportions of *Nonlabens* sp. There are no documented cases of infection by *Nonlabens* sp. in humans, however it does have a

broad antibiotic resistance profile (streptomycin, tetracycline, chloramphenicol, kanamycin, gentamicin, netilmicin, tobramycin, amikacin, ofloxacin, fosfomycin, colistin and nalidixic acid) and has been suspected to be an opportunistic pathogen like its *Flavobacteriaceae* relatives (Barbeyron *et al.*, 2011). Other potentially pathogenic taxa of interest found in the biocidal treatments include *Tenacibaculum* sp. (unidentified species and *T. Dicentrarchi),* which is an emerging pathogen in salmonid and red conger eel fisheries (Piñeiro-Vidal *et al.*, 2012; Saldarriaga-Córdoba, Irgang and Avendaño-Herrera, 2021; Echeverría-Bugueño *et al.*, 2023; Escribano *et al.*, 2023), and therefore could pose a wider ecological risk. *Aquimarina* spp. are associated with cuticular disease of lobsters and mud crabs (Yu *et al.*, 2013; Ooi *et al.*, 2020), and *Pseudoalteromonas*, which is a prolific pathogen to multiple species such as seaweed (Chen *et al.*, 2023), coral (Beurmann *et al.*, 2017), fish (Pujalte *et al.*, 2007) and molluscs (M. Li *et al.*, 2021). The VC Offshore biofilm also had a significant proportion of from *Ulvibacter* sp., which was also seen in higher relative abundances in the non-biocidal treatments. The non-biocidal treatments also contained *Aquimarina, Nonlabens* and *Tenacibaculum*, but with the addition of *Aquibacter* sp. which has been recorded as a seagrape pathogen (Kopprio *et al.*, 2021). Only the Coppercoat, Trilux (biocidal) and Silic-One (fouling release non-biocidal) biofilms contained members from the *Vibrionaceae* family, and only Coppercoat and the SilicOne biofilms contained members of the genus *Vibrio* (Figure 2.15). There are over 100 described *Vibrio* species, of which, 12 are recorded as causing human disease, and many more causing other marine diseases (Baker-Austin *et al.*, 2018). *Vibrio* spp. are often the focus of marine disease research due to its impact on commercially important fisheries and as a biosecurity risk (zu Ermgassen *et al.*, 2020; de Souza Valente and Wan, 2021; Sanches-Fernandes, Sá-Correia and Costa, 2022), as well as human-associated diseases such as *Vibrio cholerae* (Cottingham, Chiavelli and Taylor, 2003). The biofilms from Coppercoat, Primer, and Silic One also showed large proportions of *Mycobacterium*. *Mycobacterium* is a diverse group and contains many human and animal pathogenic species such as *M. marinum* (Hashish *et al.*, 2018) *, M. leprae* (Kato, 1973) and *M. tuberculosis* (Smith, 2003)*,* all of which had positive reads within the shotgun analysis.

These findings suggest that both the biocidal and non-biocidal anti-fouling coatings may harbour potentially harmful pathogens, which could pose a risk to marine life and human health, as well as commercially important fishery species. Based on the taxa

found, the types of pathogens identified were more associated with marine disease rather than human disease. It is worth noting though, the detection levels of these pathogens were relatively low and their presence does not indicate pathogenicity or immediate risk.

2.4.3 Predicted Functional Abilities and ARG Detection

Using PICRUSt2, KEGG categorisation indicated similar overall functionality across all coating types (Figure 2.16) despite the varied community compositions (Figure 2.12). The KEGGs that were identified as being differentially abundant between coating treatments related to metabolism and membrane proteins (Figure 2.17, Table 2.2). K18904 and K18904, which were in the top three predicted KEGGs, relate to an outer membrane protein multidrug efflux system, and were predicted to be abundant in the non-biocidal biofilms (Figure 2.17). When comparisons by coating were conducted (Figure 2.17, Table 2.2), KEGGs related to enzyme activity demonstrated the clearest gradient between biocidal and non-biocidal coatings. 4-hydroxybenzoate-CoA ligase (K04105) predicted abundance was highest in the Silic One coating, then Primer, Ecopower, Coppercoat, VC Offshore and then Trilux. 4-hydroxybenzoate-CoA ligase has been documented to play a role in catabolism pathways of aromatic hydrocarbons (Durante-Rodríguez *et al.*, 2018; Godínez-Pérez *et al.*, 2023), aligning with microbial species characteristics identified earlier. Three of the KEGGs identified in this analysis related to biofilm formation: K12276 K11907 and K01912. Although there was not a clear division in the biocidal treatment category and their predicted abundance, the Coppercoat biofilm had the highest abundance for all three KEGGs, while the Trilux biofilm had the lowest or second lowest abundance.

Using PICRUSt2 on the mature biofilm samples, beta-lactam KEGGs are highly prevalent. However, in the shotgun analysis, beta-lactam classes were not the most dominant; rather, polymyxin and multidrug classes were (Figure 2.25). These were not detected in the PICRUSt2 predictions as the predefined list of AMR KEGGs did not have specific polymyxin or multidrug class KEGGs, highlighting the limitation of the predictive tool. The shotgun analysis did, however, highlight the diverse range of beta-lactam classes that were present, and the variation between the coating treatments (Figure 2.27). For example, the Ecopower biofilm only had one beta-lactam class (Vibrio metallo-β-lactamase 1, VMB-1) identified, whereas the Coppercoat biofilm had 11. The biofilms from the biocidal coatings had more beta-lactam classes (24) present

overall compared to the non-biocidal coatings (16), out of the 29 classes found in total. Beta-lactamases are enzymes that have multi-resistance to beta-lactam antibiotics such as penicillins, cephalosporins, cephamycins and monobactams (Kumar, 2017). The beta-lactam ARG VMB-1 found in the Ecopower treatment, has previously been characterised within *Vibro* spp. as a carbapenemase known as Vibrio metallo-β-lactamase 1 (VMB-1) (Zheng *et al.*, 2020). *Vibrio spp.* however was not detected in the biofilm from the Ecopower coating, although it was found in the Coppercoat and Silic One biofilms. The Trilux treatment had a particularly large predicted abundance of beta-lactam-ELM1, which has been identified to be associated with *Erythrobacte*r spp. (Bahr, González and Vila, 2021). This correlates with the identification of *Erythrobacter* as a differentially abundant species in the biocidal treatments (Figure 2.14).

A highly diverse set of multi-drug class ARGs were also detected. Ecopower had a similar profile to those in the biocidal category, similar to the community composition comparisons earlier. The other two non-biocidal coatings, the primer and foul release Silic One have a much smaller contribution to its resistome from this category (Figure 2.26).

Taken together, there is no clear-cut difference in total ARGs found between biocidal and non-biocidal treatments. Copper-coat was unique in its aminoglycoside ARG component and formed a substantial element of its resistome, unlike the other treatments (Figure 2.25). Aminoglycosides include antibiotics such as gentamicin, amikacin, tobramycin, neomycin, and streptomycin (Germovsek, Barker and Sharland, 2017). The biggest difference in species composition between biofilm on Coppercoat and those of the other coatings was the abundance of *Marinobacter*, which may be driving the large aminoglycoside ARG detection; however specific isolation and screening would be needed to verify this.

2.4.4 Resistome Risk Score

Using MetaCompare2.0 and multiple metagenome assemblies (metaSPAdes, MEGAHIT and IDBA-UD), human and ecological risk were each given a resistome risk score based upon ARG and pathogen presence (based on MetaCompare2.0 database of ARGs, MRGs and pathogens). In this study, HHRR scores were low overall, ranging from zero (Ecopower idba and MEGAHIT assemblies) to 0.029 (VC Offshore MEGAHIT assembly) (*Figure 2.28*). In comparison, ERR scores ranged from 1.83 (Coppercoat MetaSPAdes assembly) to 6.08 (Trilux MEGAHIT assembly), suggesting a higher ecological health risk than human risk (*Figure 2.28*). For score comparison

against other published resistome scores, river sediment samples that were assessed in the validation of MetaCompare 2.0 produced ERR scores of ~5 for polluted river sediments and a score of ~4 for nonpolluted rover sediment. The HHRR scores produced for the same samples were ~0.5 for polluted river sediments and <0.01 for unpolluted.

There was a significant difference between the biocidal and non-biocidal HHRR scores (Wilcoxon p=0.01) based on all three assembly types (*Figure 2.28*). This indicated that the communities on the biocidal coatings contained more pathogens of interest (based on MetaCompare's ESKAPE list), ARGs and MRGs as defined by the MetaCompare protocol. ERR scores were also higher in the biocidal group, but the MetaSPAdes assembly predicted lower risk compared to the other two assembly methods, making the difference not statistically significant. However, if the MetaSPAdes assembly is removed due to its reduced compatibility with the pipeline, the differences became significant. The higher ERR scores correlate with the animal pathogen detection observations as outlined earlier in this chapter (Figure 2.15).

2.4.5 Conclusions

In this study, the dominant taxa identified across all the coatings tested are typically characteristic of polluted environments; an unsurprising result given that the coatings were deployed within a busy and working port. However, even with this driver, the coatings have distinct and unique differences demonstrating coating-specific microbial communities. Biocidal category was the clearest discriminator between coatings using beta diversity metrics, illustrating similarities between the biocidal coatings. The temporal diversity in the organisms that were identified is likely due to the resistance to the biocidal coatings, which over time resulted in a more diverse core community, albeit lower in diversity than the non-biocidal communities. This has the potential to influence subsequent settlement, although more research would be needed in relation to the specific communities in question.

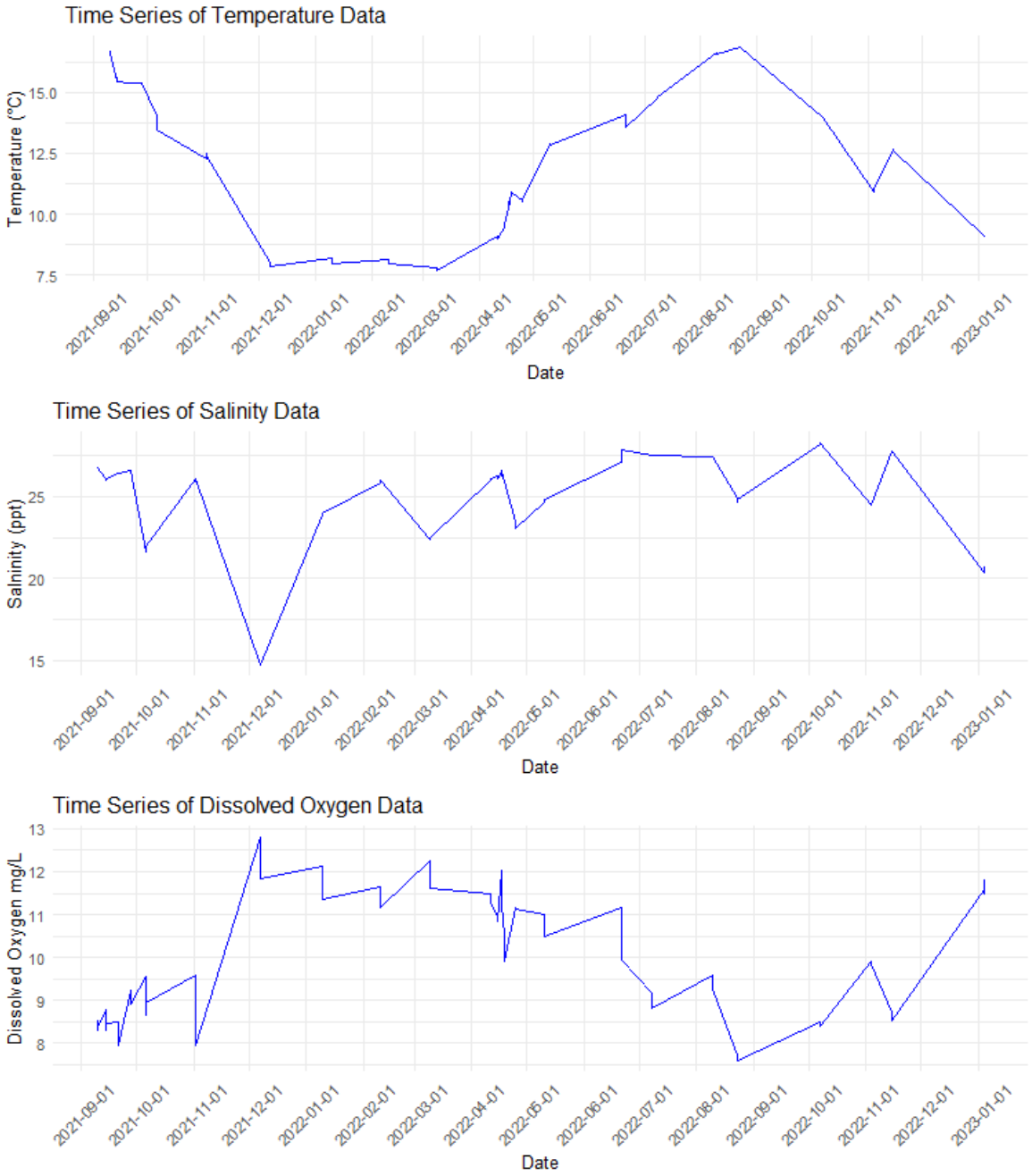
Overall, total ARG presence was not significantly different between biocidal groups, however the ARG composition revealed coating treatment differences i.e. beta-lactam classes were more diverse in the biocidal coating biofilms than those from the non-biocidal coatings. Moreover, Aminoglycoside ARGs were also well represented in the Coppercoat treatment, which was also the most taxonomically distinct.

The pathogens detected in this study are associated with marine diseases rather than human diseases. The environmental risk is on par with other polluted sites based on its ERR score, suggesting potential environmental risk. However, the presence of such species does not equate to immediate risk or pathogenicity.

2.4.5.1 Future Directions

Future planned analysis includes using the Bacmet pipeline to detect antibiotic, biocide and metal resistance genes using the shotgun dataset. Using this information, a co-occurrence network can determine the co-occurrence of resistance genes to further understand the mechanisms at play in relation to metal-based anti-fouling biocides. The profiled communities from each coating/treatment will also help inform potential microbiome acquisition of macrofoulers, which is covered in chapters 3 and 4.

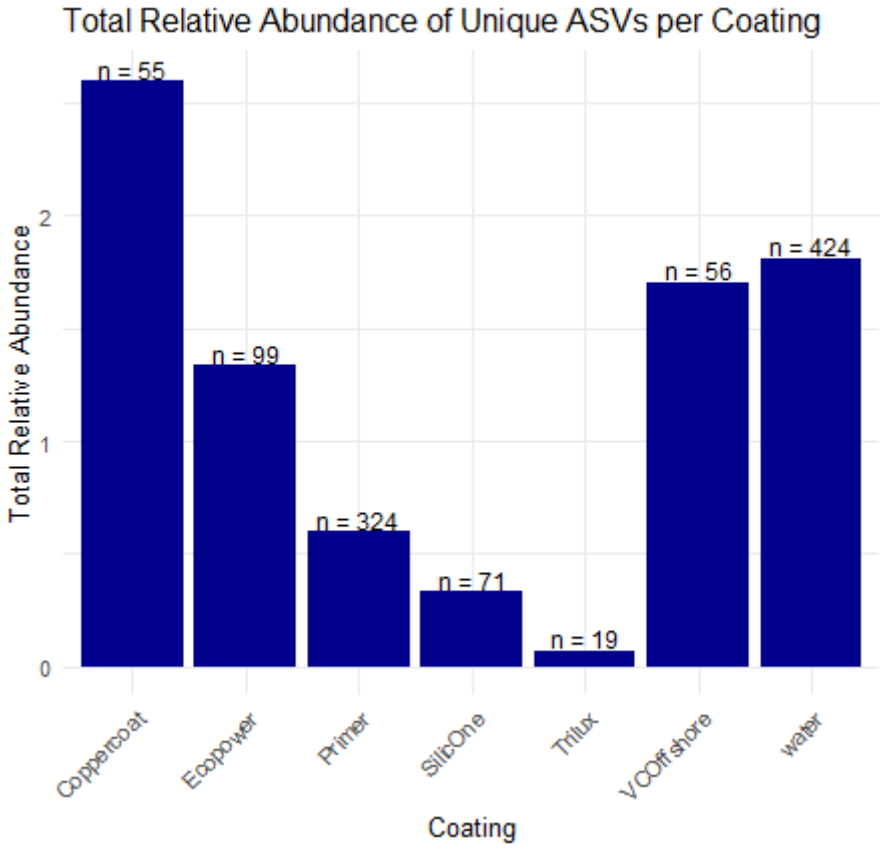
2.5 Supplementary



Supplementary Figure 2.1 Time series of environmental HORIBA data from the port of Blyth from September 2023 to January 2023.

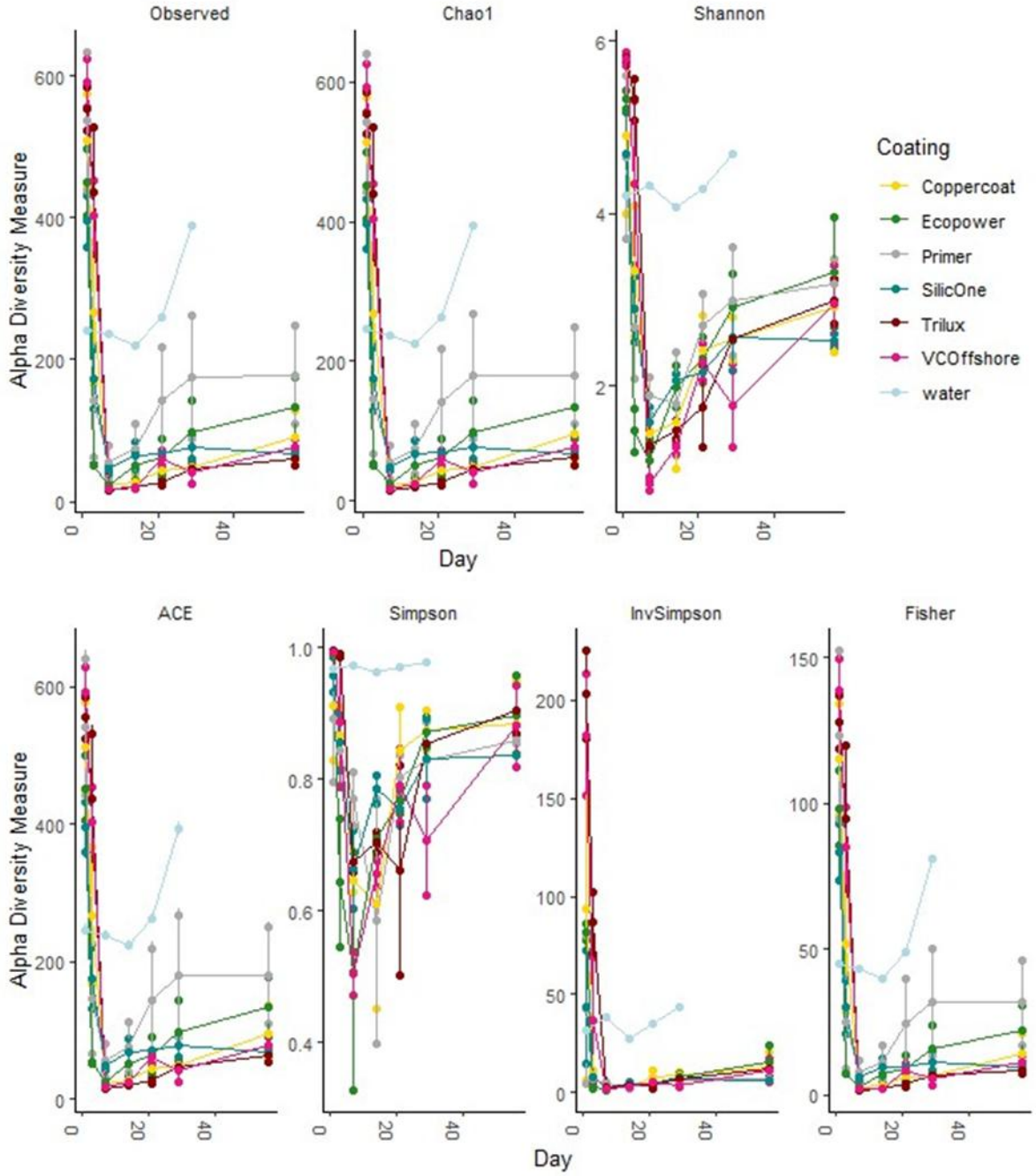
Supplementary Table 2.1 Amplicon sequencing information (16S, Bacteria, Archaea)

Sample ID	FW primer sequence	Rv primer sequence	Number of sequences	Number of sequences post taxa removal
Day29-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	83829	83445
Day7-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	77768	77479
Day56-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	76957	75548
Day14-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	73145	70340
Day7-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	70782	70347
Day7-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	64942	64695
Day14-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	53187	52663
Day56-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	49414	49338
Day56-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	48518	48091
Day29-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	46989	45733
Day29-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	44813	44440
Day7-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	44791	44251
Day1-A-water	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	44707	39842
Day14-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	44497	44378
Day7-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	44104	43787
Day7-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	43188	43072
Day56-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	43069	42640
Day21-A-water	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	42957	39328
Day14-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	42714	42624
Day29-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	41892	40893
Day29-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	41603	41371
Day3-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	41199	40347
Day56-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40651	40547
Day29-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40640	39889
Day29-A-water	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40591	37655
Day3-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40315	36105
Day21-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40306	40175
Day3-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40238	38939
Day14-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	40121	39462
Day29-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39881	39749
Day56-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39831	39340
Day21-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39788	39697
Day14-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39748	39659
Day7-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39615	39390
Day21-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39608	38117
Day3-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39564	26887
Day21-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39496	38760
Day56-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39472	39064
Day21-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	39244	39087
Day56-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38890	38648
Day1-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38814	28467
Day3-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38766	31805
Day14-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38596	38466
Day21-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38519	38389
Day14-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38477	38412
Day56-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38460	37589
Day14-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38227	38000
Day14-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38104	37816
Day7-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	38027	37182
Day7-A-water	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	37870	32399
Day14-A-water	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	37764	31673
Day29-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	37762	37374
Day21-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	37712	37023
Day7-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	37636	36913
Day29-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	37335	36862
Day3-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36954	30054
Day21-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36914	36225
Day21-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36651	32904
Day1-A-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36605	30479
Day7-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36407	36297
Day1-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36363	20031
Day7-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	36216	35753
Day7-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	35814	35673
Day56-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	35591	35171
Day3-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	35371	28667
Day56-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	35303	34860
Day29-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	35050	34938
Day1-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	34270	24862
Day29-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	34137	33857
Day1-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	33995	27327
Day21-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	33813	32801
Day14-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	33655	33482
Day14-B-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	33583	33216
Day3-A-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	33464	30148
Day21-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	33228	32889
Day1-B-Primer	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	32917	27196
Day1-B-Copper-coat	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	32672	24637
Day3-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	32413	23834
Day1-A-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	31977	18592
Day3-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	31765	31007
Day1-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	31683	23142
Day21-A-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	31637	31400
Day29-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	31171	31143
Day56-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	30239	30195
Day1-B-Ecopower	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	29771	17277
Day1-A-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	28018	19526
Day1-A-VC-Offshore	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	27960	19123
Day3-B-Silic-One	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	26999	23496
Day3-B-Trilux	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	25261	14445



1422

1423 *Supplementary Figure 2.2 Total relative abundance of unique ASVs for each mature*
1424 *biofilm coating treatment. Number of unique ASVS represented above each bar.*



Supplementary Figure 2.3 Alpha diversity metrics for each coating, over time from day 1 to day 56. From left to right: Observed Features, Chao1, Shannon, ACE, Simpson, InvSimpson and Fisher

Supplementary Table 2.2 Pairwise Wilcoxon p-adjusted (BH) results of diversity metrics of mature biofilms between grouped biocidal categories. Green filled cells indicate significant p-values <0.05.

Biocidal category		Observed	Shannon	Choa	ACE	Simpson	Fisher
group1	group2	p.adj	p.adj	p.adj	p.adj	p.adj	p.adj
Biocidal	Non-biocidal	0.001	0.134	0.002	0.001	0.767	0.001
	seawater	0.027	0.016	0.027	0.011	0.016	0.027

Non-Biocidal	seawater	0.027	0.016	0.016	0.011	0.016	0.027

Supplementary Table 2.3 Pairwise Wilcoxon p-adjusted (BH) results of diversity metrics of mature biofilms between coatings. There are no significant differences at p=<0.05

Coatings		Observed	Shannon	Choa	ACE	Simpson	Fisher
group1	group2	p.adj	p.adj	p.adj	p.adj	p.adj	p.adj
Coppercoat	Ecopower	0.252	0.864	0.305	0.342	0.864	0.252
	Primer	0.109	0.420	0.106	0.106	0.727	0.109
	SilicOne	0.115	0.773	0.115	0.115	0.396	0.115
	Trilux	0.280	0.591	0.297	0.297	0.727	0.280
	VC						
	Offshore	0.485	0.679	0.485	0.485	0.631	0.485
	water	0.115	0.250	0.115	0.115	0.250	0.115
Ecopower	Primer	0.262	0.904	0.252	0.252	1.000	0.262
	SilicOne	0.485	0.500	0.485	0.485	0.722	0.485
	Trilux	0.109	0.420	0.109	0.109	0.955	0.109
	VC						
	Offshore	0.280	0.277	0.297	0.297	0.727	0.280
	water	0.115	0.250	0.115	0.115	0.250	0.115
Primer	SilicOne	0.115	0.277	0.115	0.115	0.727	0.115
	Trilux	0.091	0.277	0.091	0.091	1.000	0.091
	VC						
	Offshore	0.109	0.277	0.106	0.106	0.727	0.109
	water	0.115	0.250	0.115	0.115	0.250	0.115
SilicOne	Trilux	0.109	0.984	0.109	0.109	0.824	0.109
	VC						
	Offshore	0.342	0.904	0.435	0.342	1.000	0.342
	water	0.115	0.250	0.115	0.115	0.250	0.115
	VC						
Trilux	Offshore	0.163	1.000	0.163	0.198	0.864	0.163
	water	0.115	0.250	0.115	0.115	0.250	0.115

VC Offshore	water	0.115	0.250	0.115	0.115	0.250	0.115

Supplementary Table 2.4 Full list of AMR related KEGGs found using PICRUST2 analysis in the whole dataset, with their corresponding threat level and associate drug group, adapted from Batool et al. (Batool et al., 2023)

#NAME	Description	threat level	Drug group
K00561	23S rRNA (adenine-N6)-dimethyl transferase	B11	Macrolide antibiotic (DG01551)
K00638	chloramphenicol O-acetyltransferase type B	B1 B6 B7	Phenicol (DG01576)
K03395	aminoglycoside 3-N-acetyltransferase I	B1 B6 B7	Aminoglycoside (DG01447)
K04343	streptomycin 6-kinase	B1 B6 B7	Aminoglycoside (DG01447)
K06979	macrolide phosphotransferase	B1	Macrolide antibiotic (DG01551)
K08217	MFS transporter, DHA3 family, macrolide efflux protein	B7 B11	Macrolide antibiotic (DG01551)
K10673	streptomycin 3"-kinase	B1 B6 B7	Aminoglycoside (DG01447)
K17840	aminoglycoside 2'-N-acetyltransferase I	B6 B12	Aminoglycoside (DG01447)
K18220	ribosomal protection tetracycline resistance protein	A1	Tetracycline (DG00005)
K18231	macrolide transport system ATP-binding/permease protein	B1 B11	Macrolide antibiotic (DG01551)

K18552	MFS transporter, DHA1 family, florfenicol/chloramphenicol resistance protein	B6 B7	Phenicol (DG01576)
K18554	chloramphenicol 3-O phosphotransferase	B12	Phenicol (DG01576)
K18698	beta-lactamase class A TEM	A3 B1 B4 B6 B7 B8 B12	Extended-spectrum cephalosporin (DG01776, DG01777),Monobactam (DG01454)
K18781	metallo-beta-lactamase class B VIM	B4 B6	Extended-spectrum cephalosporin (DG01776, DG01777),Carbapenem (DG01458)
K18790	beta-lactamase class D OXA-1	B4 B6 B7 B9	Extended-spectrum cephalosporin (DG01776, DG01777),Extended spectrum penicillin (DG01780)
K18795	beta-lactamase class A CARB-1	B6 B7	Carbenicillin (DG00519)
K18815	aminoglycoside 6'-N-acetyltransferase I	B1 B6 B7	Aminoglycoside (DG01447)
K18816	aminoglycoside 6'-N-acetyltransferase I	B1 B6 B7 B11	Aminoglycoside (DG01447)
K18845	16S rRNA (guanine(1405)-N(7))-methyltransferase	B1 B6	Aminoglycoside (DG01447)

K19097	beta-lactamase class A VEB	B1 B4 B6	Extended-spectrum cephalosporin (DG01776, DG01777)
K19209	beta-lactamase class D OXA-42		Narrow-spectrum penicillin (DG01779)
K19216	metallo-beta-lactamase class B IND		Carbapenem (DG01458)
K19217	beta-lactamase class A CARB-17		
K19218	beta-lactamase class A CARB-5	B1	Carbenicillin (DG00519)
K19271	chloramphenicol O-acetyltransferase type A	A3 B1 B6 B7 B11	Phenicol (DG01576)
K19272	aminoglycoside 3'-phosphotransferase I	B1 B7	Aminoglycoside (DG01447)
K19278	aminoglycoside 6'-N-acetyltransferase Ib	B1 B6	Aminoglycoside (DG01447)
K19299	aminoglycoside 3'-phosphotransferase III	B11	Aminoglycoside (DG01447)
K19300	aminoglycoside 3'-phosphotransferase II	B6	Aminoglycoside (DG01447)
K19301	aminoglycoside 6'-N-acetyltransferase II	B1 B6	Aminoglycoside (DG01447)
K19319	beta-lactamase class D OXA-134	B1	Carbapenem (DG01458)
K19321	beta-lactamase class D OXA-214	B1	Extended-spectrum penicillin (DG01780) Carbapenem (DG01458) (weak)

1439 (Batool *et al.*, 2023)

Threat level by CDC

A. Urgent threats

A1. Clostridium difficile

A2. Carbapenem-resistant Enterobacteriaceae (CRE)

A3. Drug-resistant Neisseria gonorrhoeae

B. Serious threats

B1. Multidrug-resistant Acinetobacter

B2. Drug-resistant Campylobacter

B3. Fluconazole-resistant Candida (a fungus)

B4. Extended-spectrum beta-lactamase producing Enterobacteriaceae (ESBLs)

B5. Vancomycin-resistant Enterococcus (VRE)

B6. Multidrug-resistant Pseudomonas aeruginosa

B7. Drug-resistant non-typhoidal Salmonella

B8. Drug-resistant Salmonella enterica serovar Typhi

B9. Drug-resistant Shigella

B10. Methicillin-resistant Staphylococcus aureus (MRSA)

B11. Drug-resistant Streptococcus pneumoniae

B12. Drug-resistant tuberculosis

C. Concerning threats

C1. Vancomycin-resistant Staphylococcus aureus (VRSA)

C2. Erythromycin-resistant group A Streptococcus

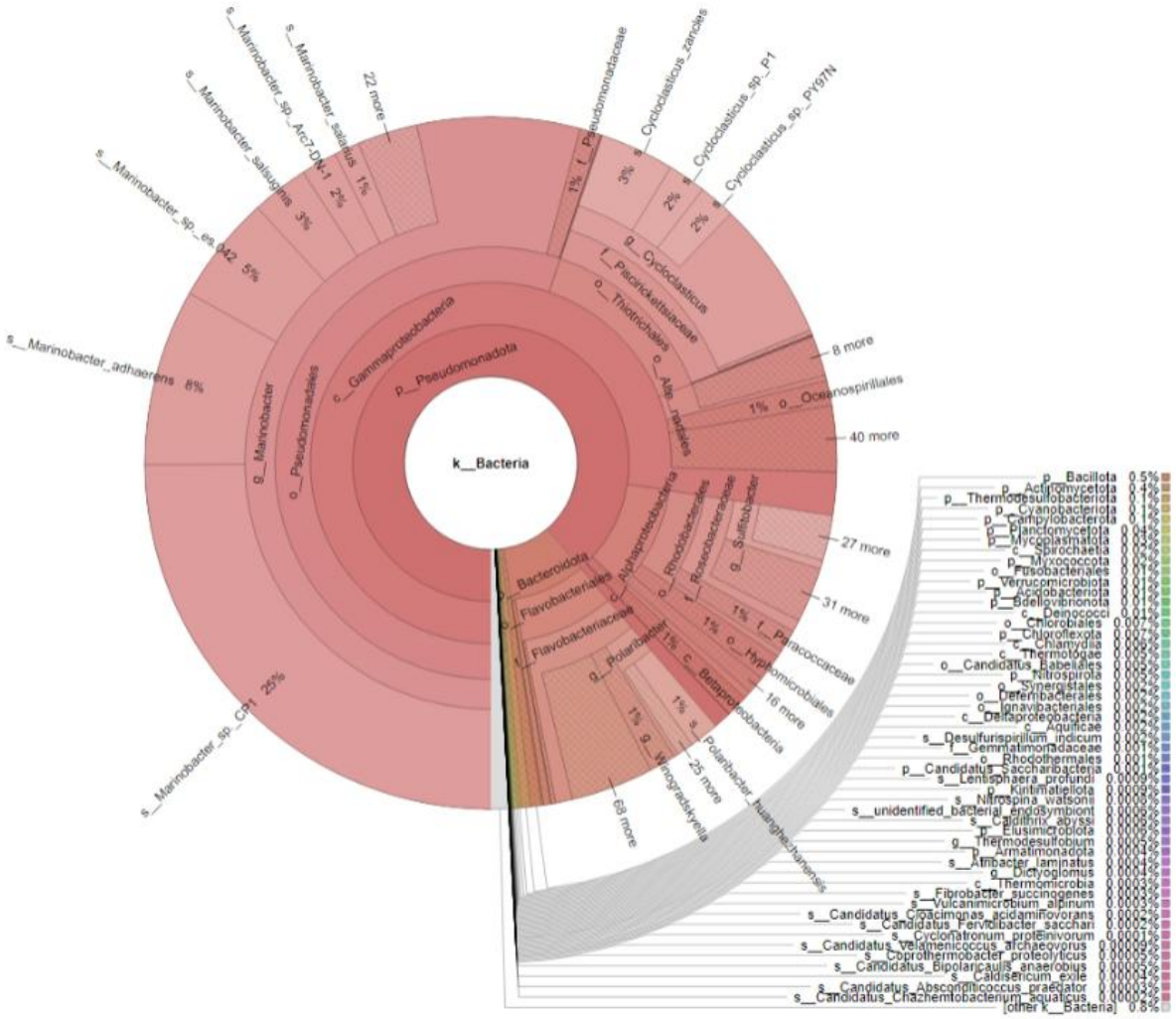
C3. Clindamycin-resistant group B Streptococcus

1440

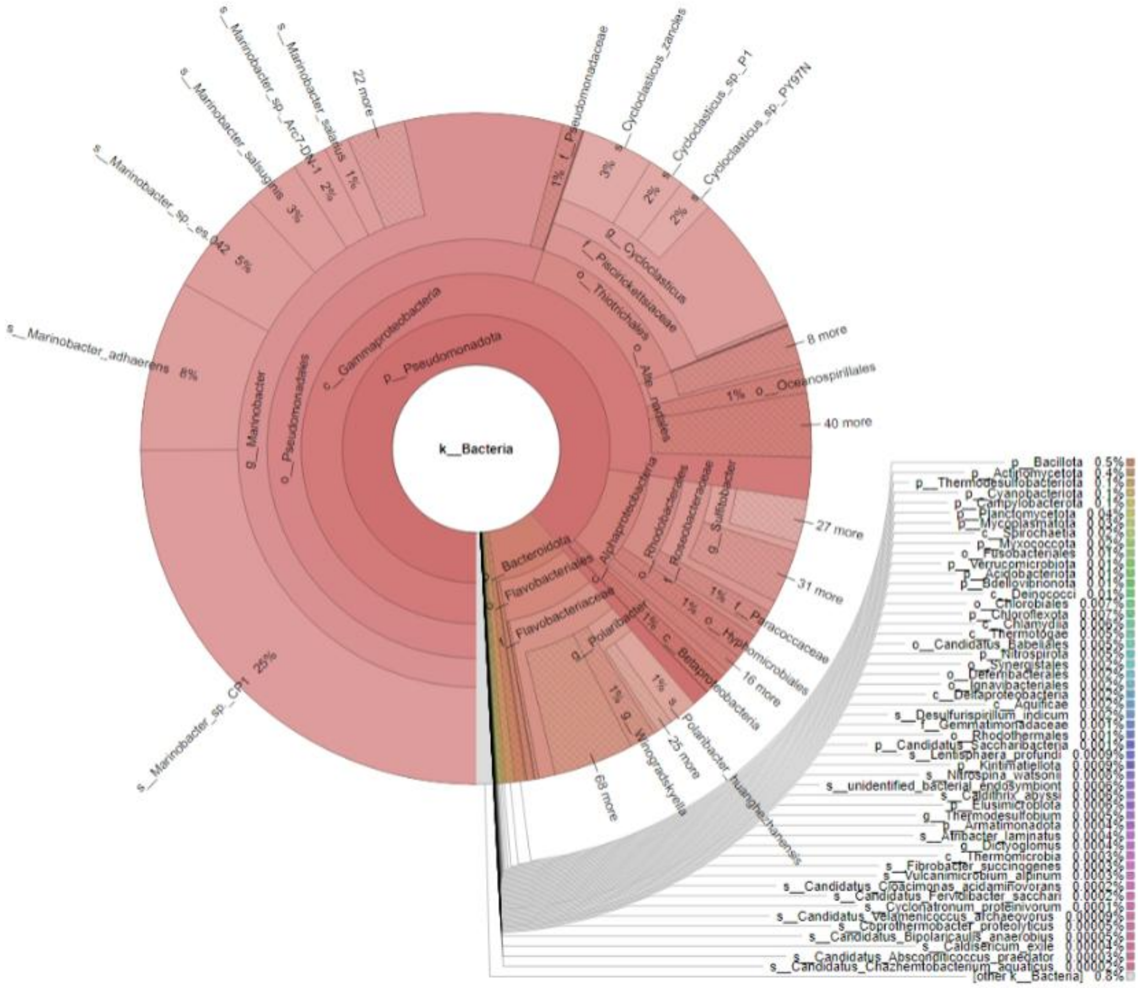
1441

1442 *Supplementary Table 2.5 Pavian Kraken Report summary*

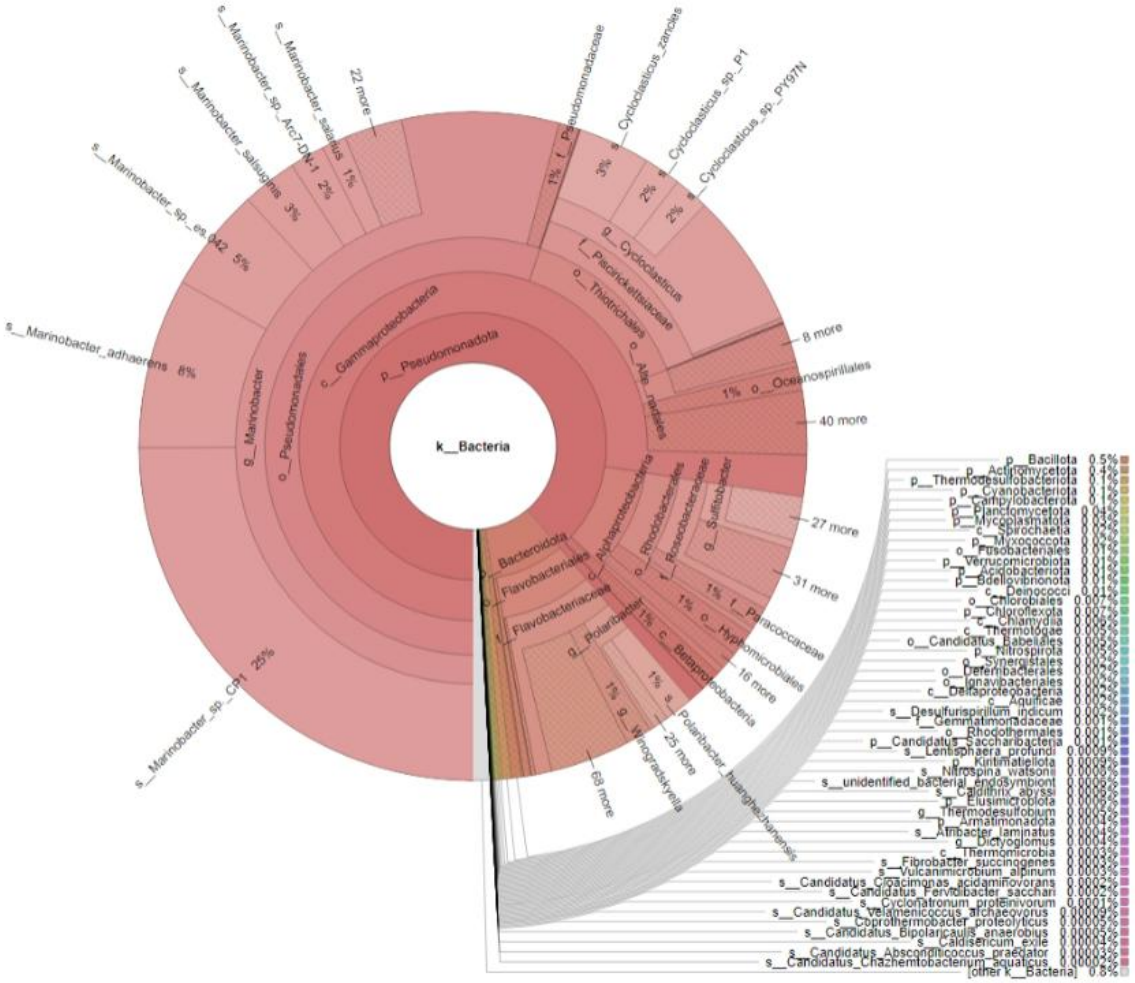
Name	Number of raw reads	Classified reads	Chordate reads	Artificial reads	Unclassified reads	Microbial reads	Bacterial reads	Viral reads
COP	82,678,113	51.8%	0.692%	0%	48.2%	51.1%	51.1%	0.0181
ECO	32,356,415	30.3%	0.736%	0%	69.7%	29.5%	29.5%	0.00812
PRI	78,602,214	25.9%	0.785%	0%	74.1%	25.1%	25%	0.0121
SIL	76,098,450	46.2%	0.566%	0%	53.8%	45.6%	45.5%	0.0114
TRI	54,662,117	45.6%	0.563%	0%	54.4%	45%	45%	0.012
VC	427,262	44.7%	0.627%	0%	55.3%	44%	44%	0.0115



Supplementary Figure 2.4 Krona plot for Coppercoat mature biofilm sample.

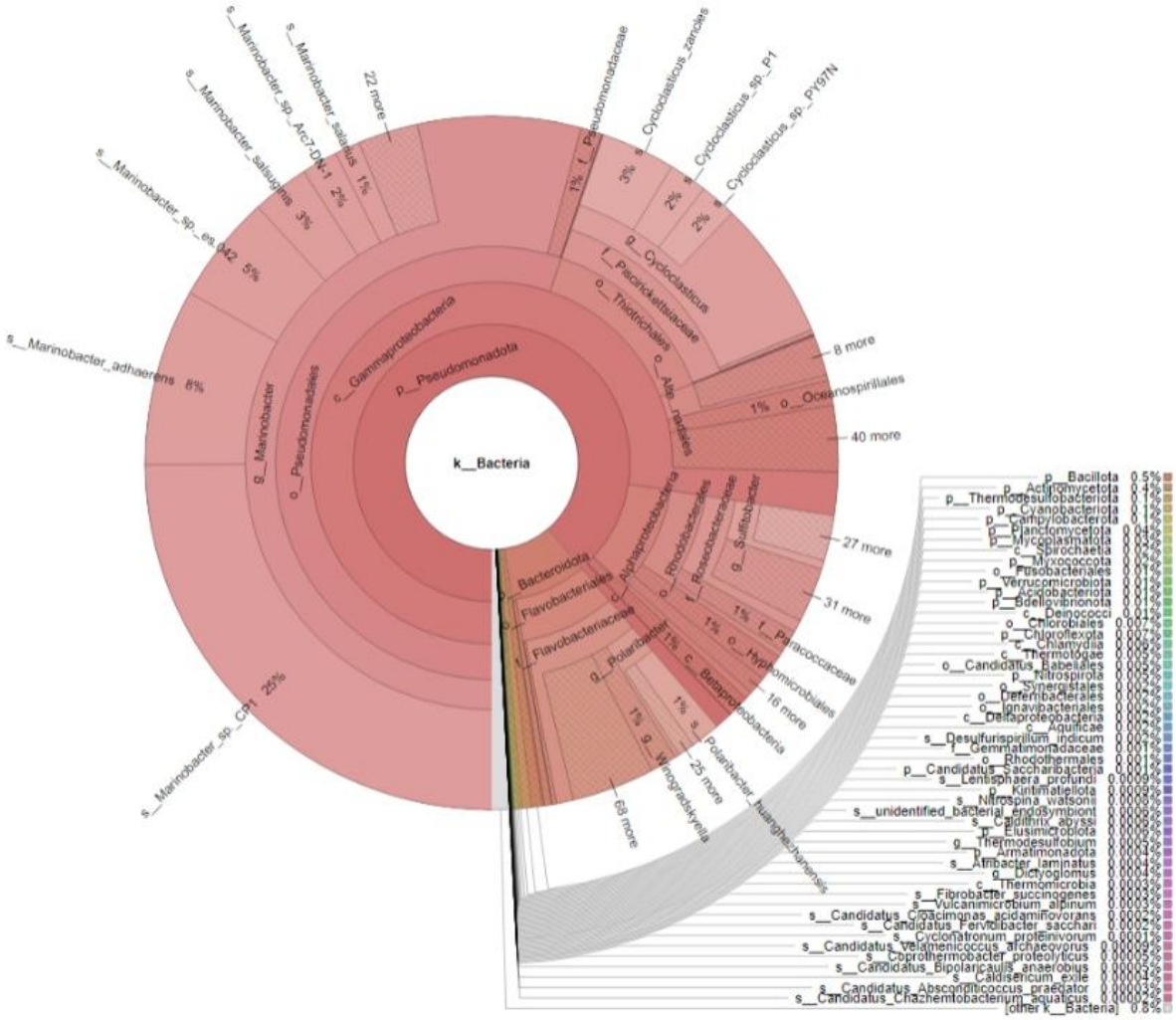


Supplementary Figure 2.5 Krona plot for Ecopower mature biofilm sample.



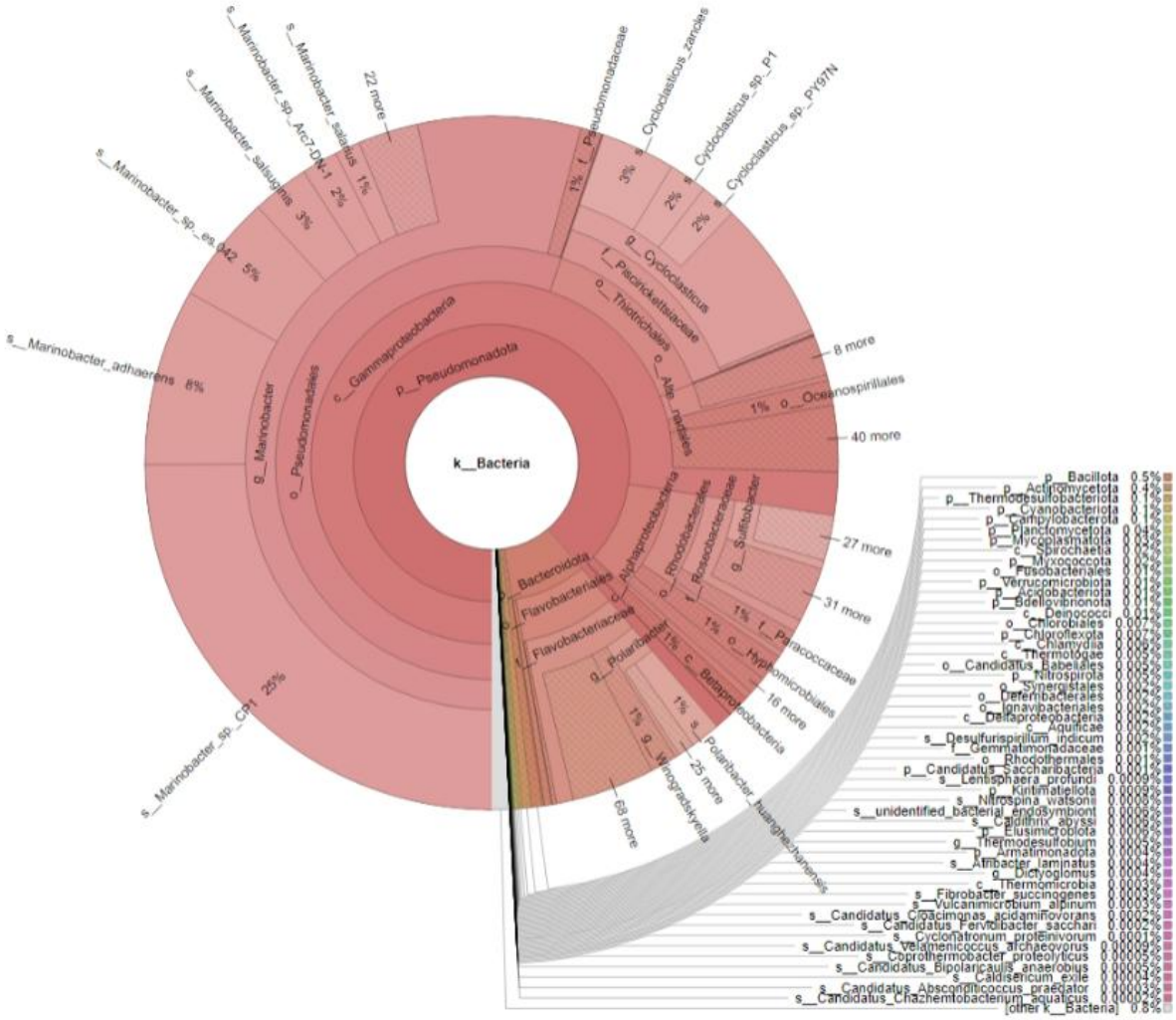
1448

1449 *Supplementary Figure 2.6 Krona plot for Primer mature biofilm sample.*

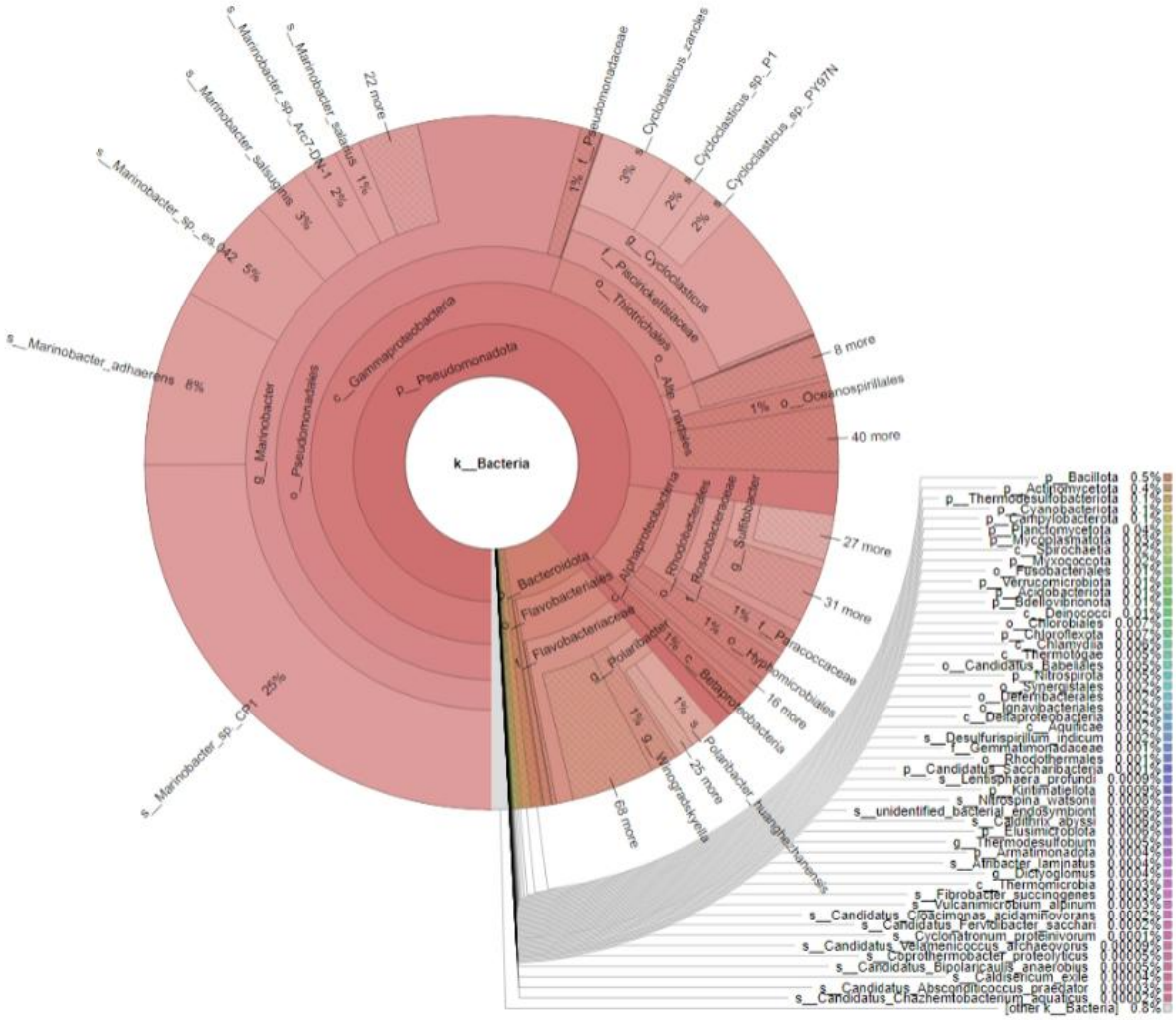


1450

1451 *Supplementary Figure 2.7 Krona plot for Trilux mature biofilm sample.*



Supplementary Figure 2.8 Krona plot for VC Offshore mature biofilm sample.



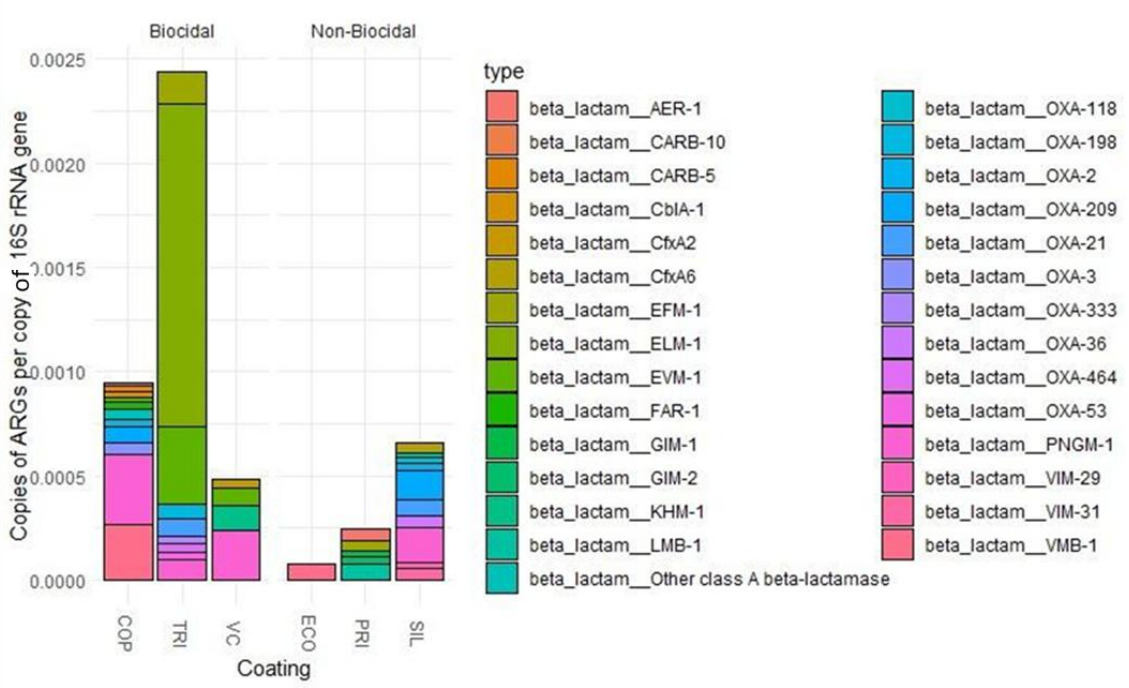
1454

1455 *Supplementary Figure 2.9 Krona plot for Silic One mature biofilm sample.*

1456 *Supplementary Table 2.6 ABRicate results based on MEGARES and VFDB results*

#COATING	APH3-DPRIME	APH6	DFRA6	QACK	SUL1	acpXL	mbtH-like	pilG	pilT	pilU
COP	100	100	81.01	100	100	92.41	.	81.37	92.37	.
TRI	.	.	.	100	.	93.25;91.14	.	81.37;81.37	99.61;92.37	.
VC	.	.	.	100	.	92.41;93.25	.	81.37	92.37	.
ECO	.	.	.	100	.	92.41	.	.	.	.
PRI	.	.	.	99.7	.	92.41;92.41;88.61	.	.	.	.
SIL	.	.	.	100	.	92.41	97.26	82.84	.	93.21

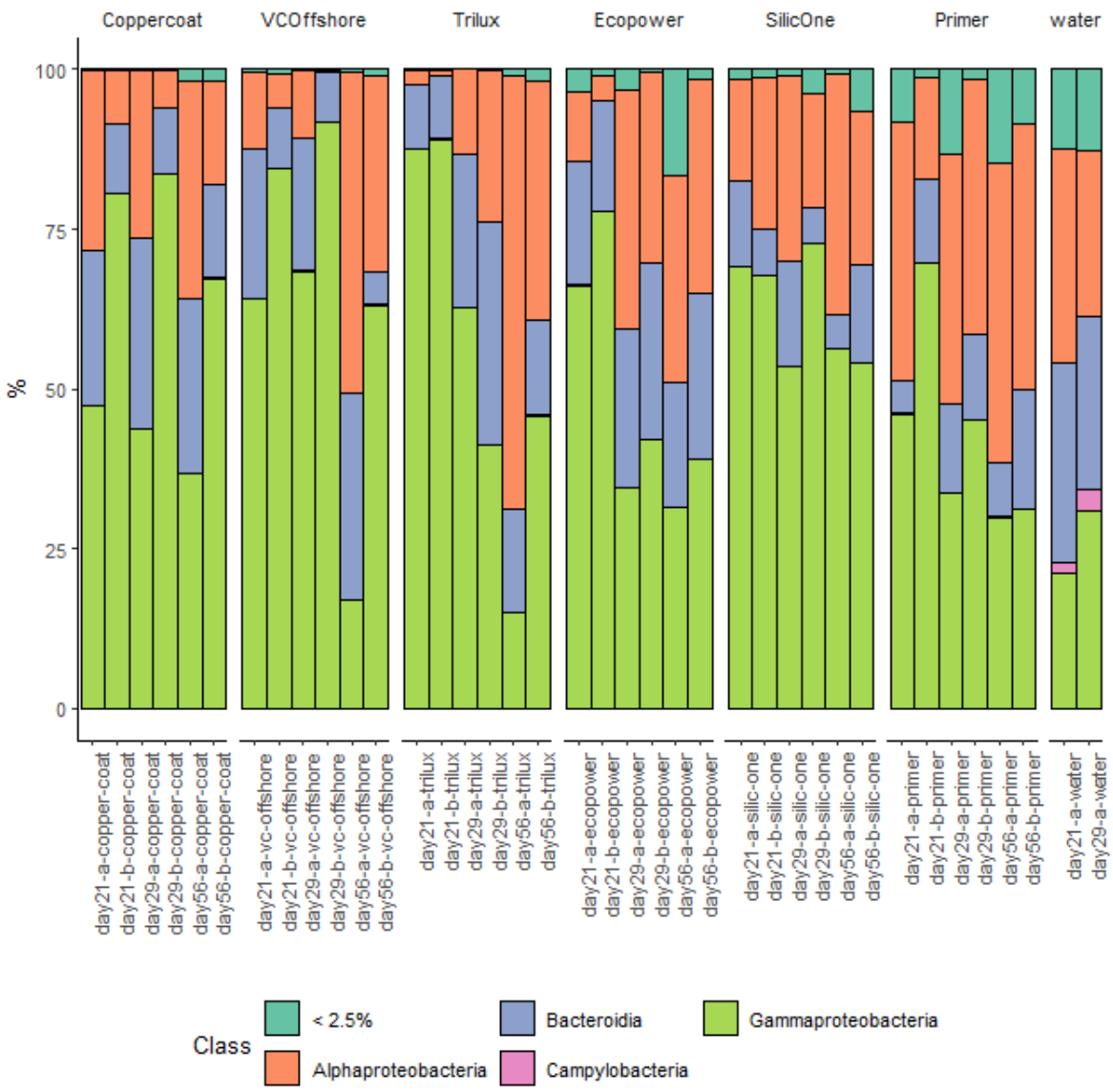
1457



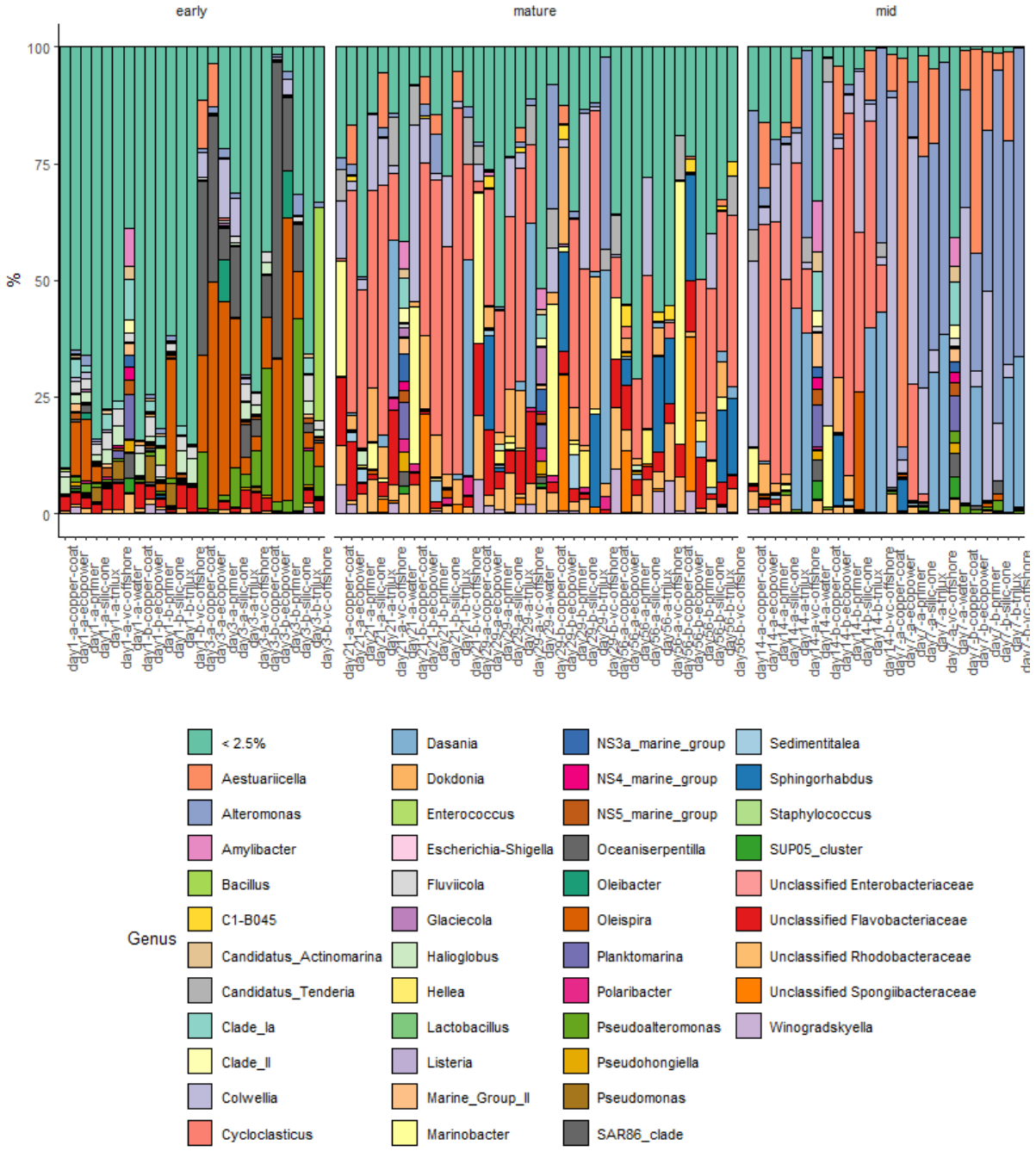
Supplementary Figure 2.10 Beta-lactam subcategories copies per copy of 16S rRNA gene found in each coating treatment

Supplementary Table 2.7 Paint composition in percentages. Ingredients in red are the active biocidal components.

Compound	VC Offshore	SilicOne	Ecopower	Primer	Trilux	Coppercoat
Reaction mass of: Xylenes and Ethylbenzene	≥25 - ≤50				≥25 - ≤30	
Zinc oxide	≥20 - <25				≥10 - ≤20	
Rosin	≥5 - ≤10				≥10 - ≤20	
n-butyl acetate		Not Avail				
2-Pentanone, O,O',O"-(ethenylsilylidyne) trioxime		Not Avail				
Octamethylcyclotetrasiloxane		Not Avail				
Solvent naphtha (petroleum), light arom			≥10 - ≤25	≤5		
Titanium dioxide			≥10 - ≤25			
4-methylpentan-2-one			≤3			
Oleic acid, compound with (Z)- N-octadec-9-enylpropane1,3-diamine (2:1)			<1			
2,5-di-tert-butylhydroquinone			≤0.3			
Trimethylolpropane			≤0.3			
(Z)-N-9-octadecenylpropane1,3-diamine			≤0.062			
Aromatic hydrocarbons, C9				≥25 - ≤50		
Solvent naphtha (petroleum), medium aliph.				≤5		
Copper thiocyanate					≥10 - ≤15	
Pyrithione zinc					≤3.5	
Copper oxide	20					95
Bisphenol A/F- Epoxy Resins MW < 700						30-60
Oxirane, mono [(C12-14- alkyloxy)methyl]derivs						5-10



Supplementary Figure 2.11 Mature community composition by Class taxonomic level.



Supplementary Figure 2.12 Community composition by genus level, ordered by 'maturity' level. Early: day1-3, mid: day7-14, mature: day21-56.

Chapter 3. Anti-fouling Coatings Influence Microbiome Acquisition of Settling *Semibalanus balanoides*

3.1 Introduction

Marine microbiomes, whether associated with a host, or the environment, are often highly diverse and play crucial roles in ecological dynamics and function. As previously discussed (Chapter 1), biofilm composition and function can heavily influence the settlement and behaviour of some, but not all, fouling invertebrates (Scheltema, 1974; Wieczorek and Todd, 1998; Olivier *et al.*, 2000; Huang and Hadfield, 2003; Dahms, Dobretsov and Qian, 2004; Hung *et al.*, 2005; Hadfield, 2011; Whalan and Webster, 2014; Vinagre *et al.*, 2020). Microbial communities associated with hosts, such as marine invertebrates, can be involved in symbiotic relationships and play significant roles in the host's health and development (Sipe, Wilbur and Cary, 2000; Lopanik, Lindquist and Targett, 2004; Fan *et al.*, 2013; Cavalcanti *et al.*, 2020; Sehnal *et al.*, 2021). In the case of coral larvae, the ability to metamorphose after settlement was greatly reduced when a tetrabromopyrole-producing bacteria was removed (Alker *et al.*, 2023). Though relatively understudied thus far, host-associated microbiomes have been demonstrated to be relatively stable (van de Water *et al.*, 2018; Brown, Nunez and Rand, 2020), however, the stasis can be disrupted and influenced by environmental stressors and disease (Hirose *et al.*, 2004; Salerno *et al.*, 2005; Fan *et al.*, 2013; Lokmer and Wegner, 2015). Disruption to the 'normal stasis' of the microbial communities associated with hosts may provide gateways for opportunistic pathogens and reduced host health (Jurelevicius *et al.*, 2021).

In the only studies to examine the stability of the microbiome of the barnacle *Semibalanus balanoides,* both temporal and geographic stability were demonstrated (Aldred and Nelson, 2019; Brown, Nunez and Rand, 2020). Aldred and Nelson (2019) showed that although their microbiomes may remain relatively stable between years, the microbiome is likely acquired from the benthos, and develops between each development stage during settlement. *S. balanoides* life cycle involves nauplii release, which goes through 6 ecdyses stages before developing into a planktonic cyprid. There is a short window of settlement (~2 weeks) during the cyprid stage, which, upon settlement, they quickly metamorphoses into a juvenile barnacle. The settling cyprids adopted a specific sub-set of benthic bacteria distinct from its planktonic predecessor and the local benthos.

As different anti-fouling coatings vary in their associated microbial communities (Chapter 2), this chapter aims determine if biocidal coatings influence the microbiome acquisition of settling Semibalanus balanoides cyprids.

3.2 Methods

3.2.1 Sample Collection

The experiment was conducted during the *S. balanoides* settlement season; 15th-25th April 2022. Samples were collected from anti-fouling coating matrix boards deployed at Blyth Marine Station, Blyth, UK (55°07'32.2"N 1°29'51.3"W) (Figure 3.1). The settlement boards had a mixed matrix of the following coatings (as in Chapter 2): non-biocidal; Hempel light primer, Hempel Ecopower Cruise, and Hempel Silic one (Foul release coating) (Hempel group, Lyngby, Denmark). Biocidal coatings include; International Trilux, International VC Offshore, (International Paint, Gateshead, UK) and Coppercoat (Aquarius Marine Coatings, Shillingstone, UK). The settlement boards from Chapter 2 were also used (Figure 2.1).

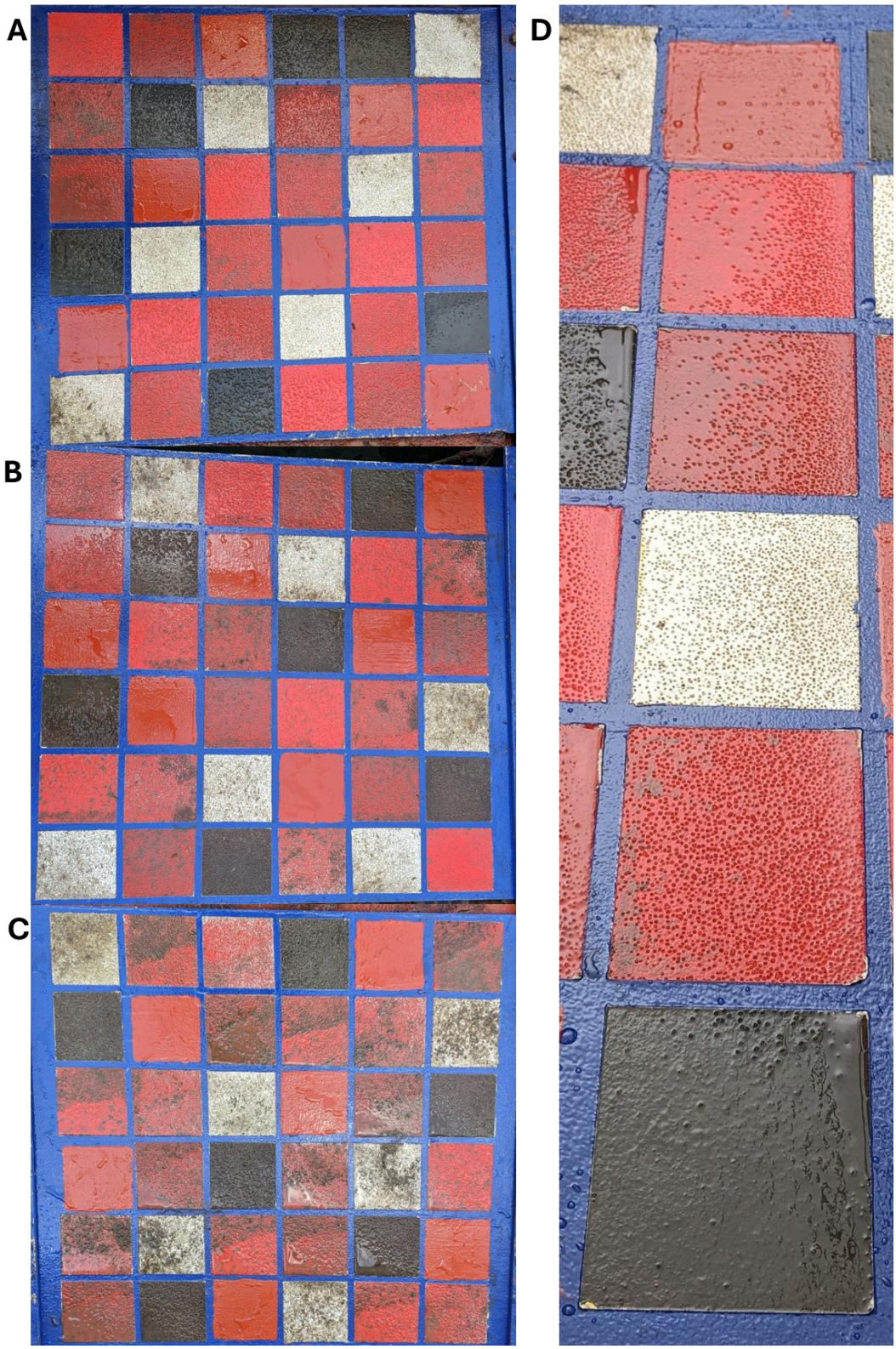


Figure 3.1 (A-C) settlement matrix boards at the port of Blyth study site with S. balanoides settlement. (D) Close up of one row from the settlement boards with visual cyprid settlement across the coatings. From top down: Silic One, Trilux, VC Offshore. Primer, Ecopower, Coppercoat.

Cyprids were collected from the water column using a 100µm plankton net, of which sixty were selected for further analysis. Cyprids collected from the final plankton trawl

were washed thoroughly using five changes of autoclaved 0.22µm filtered seawater prior to selection. Settled individuals were collected at the following life stages where feasible: (1) cyprid attachment (settled) (2) post-metamorphosis (3) calcifying/calcified (Figure 3.2). Not all life stages were present on all coatings. Individuals were collected using sterile mounted needles, one per surface type, and life stage. Thirty individuals per replicate (2-3 replicates) of each life stage were collected on the six different coatings with a replicate taken 3m apart at the same water height. Two replicates were also collected from the wooden pier adjacent to the settlement panels for a natural comparison. Swabs were taken from each surface type for benthos comparison. All samples were frozen within two hours of collection until processing for up to six months. Environmental data (salinity, temperature and dissolved oxygen) were collected using a HORIBA multiparameter meter (HORIBA Ltd, Japan) (Supplementary Figure 3.1).

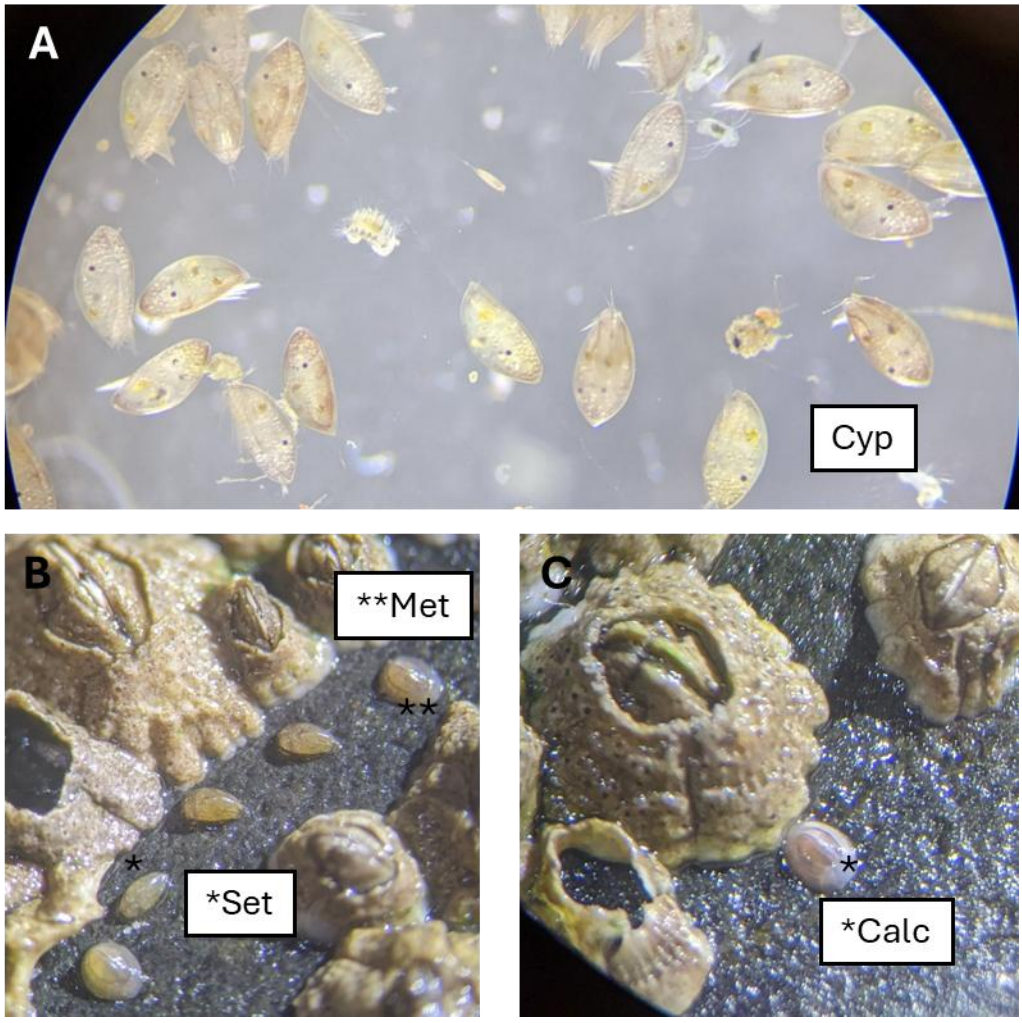


Figure 3.2 S. balanoides settlement stages collected from a rock in Seaton Sluice during exploratory field investigations. (A) planktonic cyprid (cyp). (B) Settled cyprids (Set) on a rocky substrate. Metamorphosed (Met) juvenile S. balanoides on rocky substrate. (C) juvenile calcified (Calc) S. balanoides on a rocky substrate.

3.2.2 DNA Extraction

DNA was extracted from the samples (20 cyprids per sample) using a QIAGEN DNeasy Powerlyzer Powersoil kit (QIAGEN) as per the manufacturer's instructions but with an additional wash step using ice-cold 80% ethanol prior to elution. Samples were eluted in a total volume of 50µl elution buffer. DNA was quantified using Nanodrop and prepared for 16S sequencing at NUOMICs (Northumbria University).

3.2.3 PCR Amplification and Amplicon Sequencing of 16S rRNA

PCR was performed using the Earth Microbiome Project primer set 515F–806R, which targets the V4 region of the 16S SSU rRNA (FWD:GTGYCAGCMGCCGCGGTAA (Parada, Needham and Fuhrman, 2016); REV:GGACTACNVGGGTWTCTAAT (Apprill *et al.*, 2015). Using the pre-barcoded primers (Integrated DNA Technology), a PCR was run under the following conditions: 94°C for 3 minutes, followed by 35 cycles of: 94°C for 45s, 50°C for 60s, 72°C for 90s, then a final extension period of 72°C for 10 minutes. PCR was carried out using 0.8x KAPA 2G Robust Taq polymerase (Roche), 0.2µM of each primer and 1µl of DNA. The PCR products were measured using PicoGreen (Invitrogen) on a Spark plate reader (TECAN), then normalised to a concentration of 50nmol/µl. The library was made by pooling 5µl from each sample together prior to clean up using AMPure XP (Beckman Coulter), followed by two washes using 80% ethanol. The library was resuspended in molecular grade water. Library concentration was remeasured using the high sensitivity DNA kit (Invitrogen) using the Qubit 4.0 (Invitrogen) and the product size was checked using the high sensitivity DNA kit (Agilent) on the Agilent Bioanalyzer 2100. The library was dissociated into single strand DNA using an equal volume of 0.2N sodium hydroxide which was stopped after 5 minutes using 400nM Tris pH 8.0 buffer. The library was then diluted to 20pMol with Illumina hybridisation buffer before being spiked with 20% v/v PhiX (phage lamda DNA) as an internal quality control. The final library was loaded into the Illumina MiSeq v2 500 cycle reagent cartridges.

3.2.4 Qiime2

Pre-demultiplexed fastq files were processed using the Qiime2 package, version 2023.2 (Figure 2.2) (Bolyen *et al.*, 2019). Sequences were quality checked, trimmed and denoised using DADA2 (Callahan *et al.*, 2016) with reads truncated to f-210 r-13-187 based on the 25th percentile above a quality score of 30 (3876 unique features over 51 samples/806,670 reads). Chimeras were identified and removed using UCHIME (Edgar *et al.*, 2011). Features with very low abundance below 10 reads were

also removed. Amplicon Sequence Variants (ASVs) were created based on the remaining reads and assigned taxonomies by aligning sequences to the v4 region of the 16S rRNA gene sequences from the SILVA v138 database (Quast *et al.*, 2013). Non-desirable sequences were removed that were classified as eukaryote, mitochondria, archaea, chloroplast or unknown according to SILVA taxonomies. A phylogenetic tree was created using Fasttree for alpha diversity metrics. The feature tables and taxonomies were exported and visualised using R (version 4.3.1, R Core Team, 2023).

3.2.5 R Analysis and Statistics

Potential contamination identified in the negative controls was removed using SCRuB (Austin *et al.*, 2023) (total reads per sample: Supplementary Table 3.1). Rarefaction and alpha diversity metrics were conducted using the community ecology package Vegan (Oksanen *et al.*, 2024). Samples were rarefied to 4005 reads to include as many samples as possible for alpha diversity analyses. Pairwise comparisons were conducted using the Wilcoxon rank sum exact test on alpha diversity metrics and the Holm p-value adjustment method. Beta diversity and associated distance plots were conducted through Phyloseq (McMurdie and Holmes, 2013). Sample differences were compared using analysis of similarities (ANOSIM) and pairwise PERMANOVAs. Differential analysis was conducted using LEfSe (Cao *et al.*, 2022) and DESeq2 (Love, Huber and Anders, 2014). Core ASVs were identified as those shared across all samples at either 70 or 50% prevalence.

3.3 Results

3.3.1 Unique ASVs

Unique ASVs were identified using VENN analysis (Figure 3.3). Although there is a large degree of overlap between all treatments (Figure 3.3 A-C) and life stages (Figure 3.3D), each category also had a substantial number of unique ASVs. Non-biocidal coatings contained the most total ASVs, 77% of which, were unique to that category. The planktonic cyprid had the smallest total (71), of which 32% were unique. The metamorphosed stage of *S. balanoides* had the smallest relative proportion of unique ASVs at 20%, sharing large amounts with the settled stage of development (Figure 3.3D). However the overall relative abundance of the unique ASVs per coating was relatively low, ranging from 0.13% in the VCOffshore settled samples, to a more substantial 3.5% in the wood settled samples (Figure 3.3E).

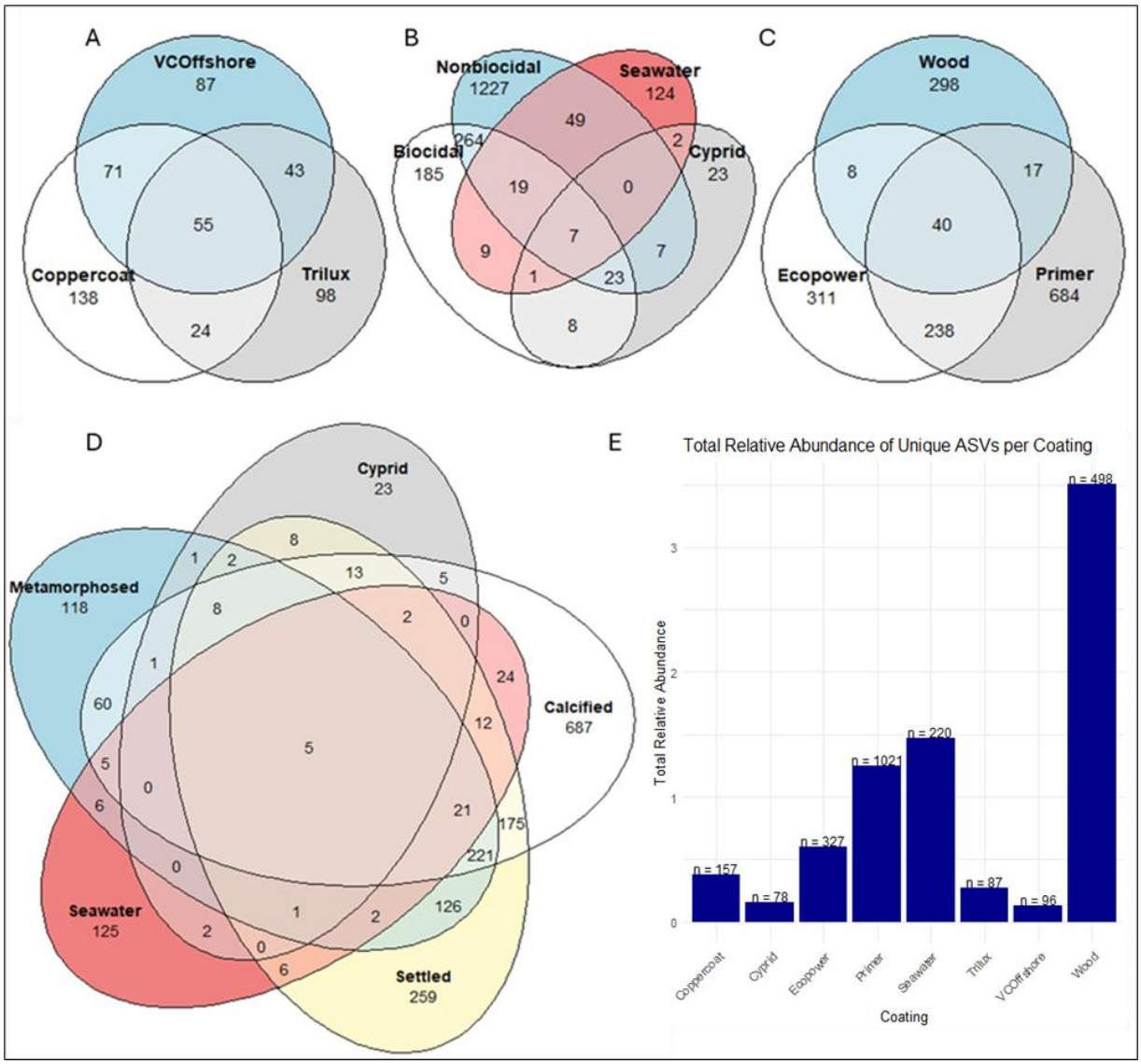


Figure 3.3 Venn diagram of unique ASVs found per treatment (A-C) and life stage (D). There is a significant level of overlap between all treatments and life stages, however, there remains a substantial number of unique ASVs found in each treatment/stage. (E) Total relative abundance of unique ASVs per Coating

3.3.2 Community Diversity

The settlement stages that had consistent significant differences were the metamorphosed and settled stages vs the calcified stage (Obs, Chao, Ace and Fisher) (Figure 3.4, Table 3.1), although potentially skewed by one calcified sample from the primer coating. Metamorphosed and settled stages vs the planktonic cyprid were also significantly different in the Chao and Ace metrics. No differences were found in any group with Shannon, Simpson or InvSimpson at 95% confidence limits (Table 3.1, Supplementary Figure 3.2, Supplementary Figure 3.3). Overall, species richness and evenness were similar, with the cyprid stage having particularly low richness.

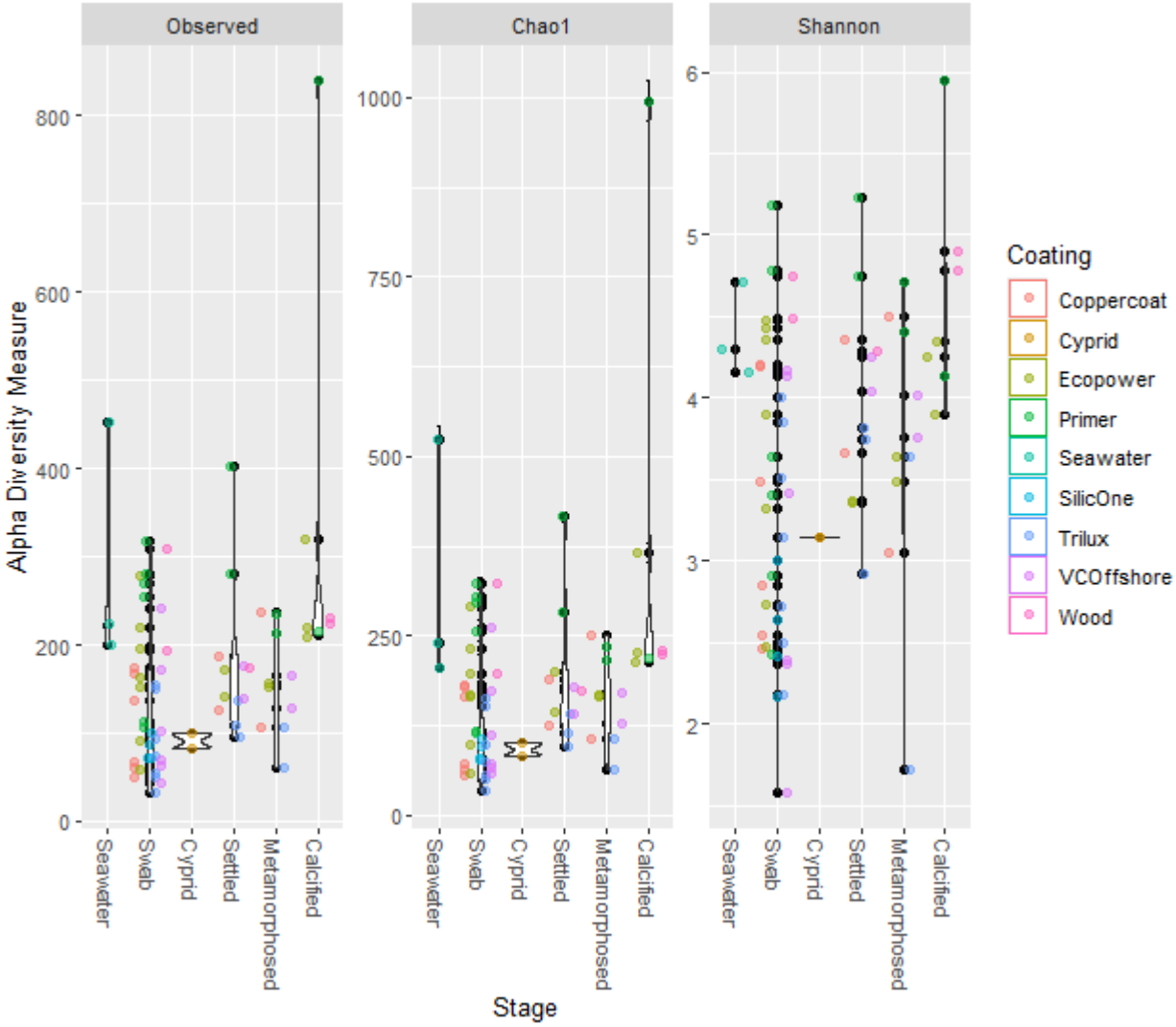


Figure 3.4 Alpha diversity (species richness) metrics for each settlement stage. Right to left: Observed, Chao1, Shannon

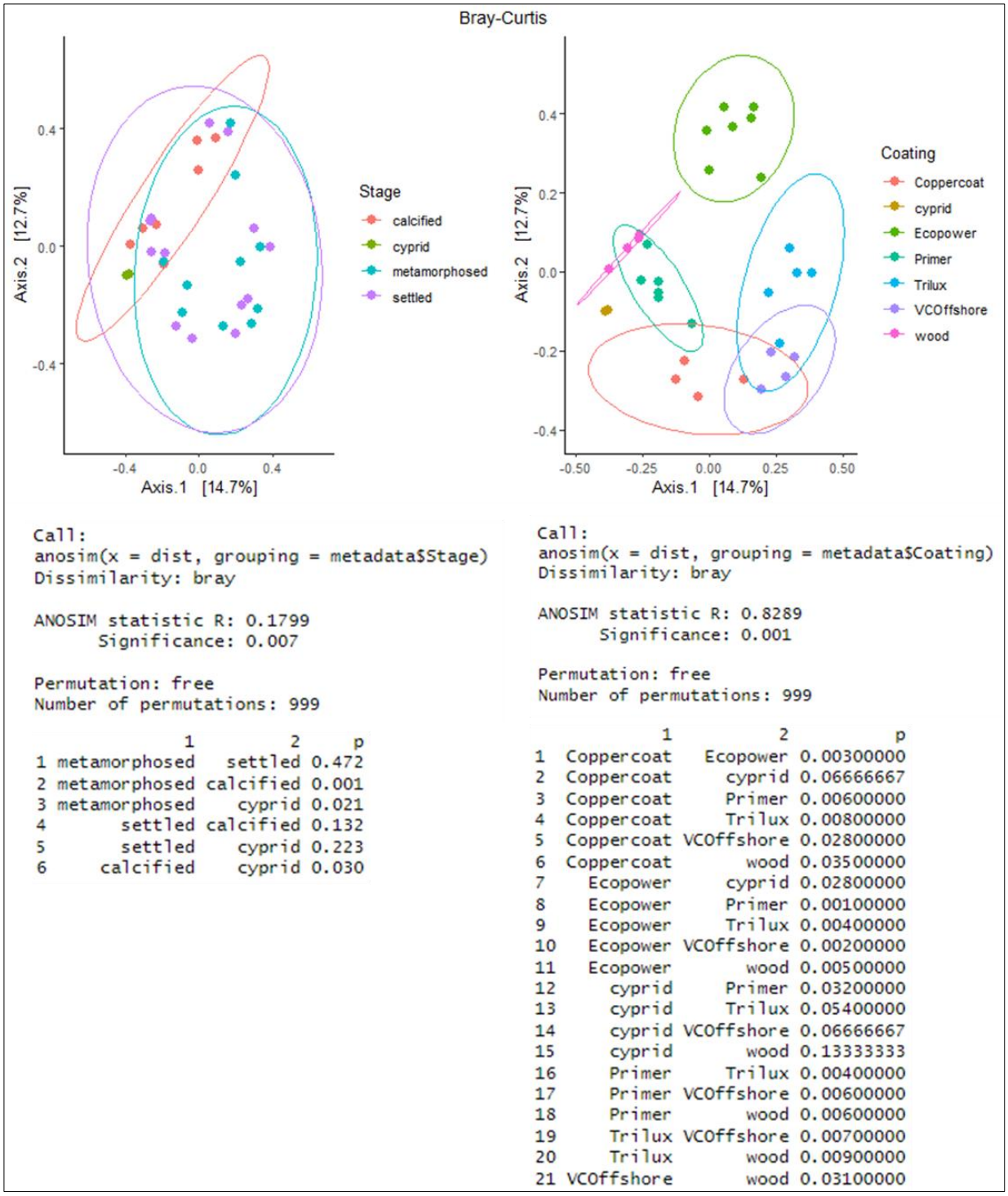
Table 3.1 Alpha diversity pairwise Wilcox results with Semibalanus development stages only. P-values highlighted in blue indicate significant p values <0.05

	Group1	Group2	Observed	Shannon	Chao1	ACE	Simpson	Fisher
			p.adj	p.adj	p.adj	p.adj	p.adj	p.adj
1	Cyprid	Calcified	0.067	0.103	0.067	0.067	0.333	0.067
2	Metamorphosed	Calcified	0.038	0.058	0.039	0.029	0.399	0.038
3	Settled	Calcified	0.038	0.103	0.039	0.029	0.409	0.038
5	Metamorphosed	Cyprid	0.061	0.327	0.045	0.045	0.409	0.061
6	Settled	Cyprid	0.061	0.066	0.044	0.044	0.422	0.061
9	Settled	Metamorphosed	0.817	0.582	0.872	0.872	0.539	0.817

3.3.3 Beta Diversity

Although species richness was similar across settlement stages, beta diversity (the differences between samples) revealed more about the community distinctions. Bray-Curtis dissimilarity PCoA plots showed clear differences between the microbial communities by coating (Figure 3.5). The Coppercoat, VC Offshore and Trilux biocidal treatments grouped together, although with clear shifts and statistical pairwise differences between individual coatings (ANOSIM R=0.8289, p=0.001) (Figure 3.5). When grouped by settlement stage, settled and metamorphosed samples overlapped (Figure 3.5), however, the calcified stages (from non-biocidal treatments only) presented a narrow band of dissimilarity. The primer and wood samples appeared to overlap, with their calcified samples grouping together. In contrast, the anti-fouling Ecopower coating, is presented in its own ordination, close to that of the other Ecopower samples. The strong grouping of samples from each coating demonstrated the major influence coating type had on the colonising microbiome.

Considering the non-biocidal treatments only, which contain all settlement stages to calcification, beta diversity (Bray-Curtis dissimilarity) is grouped distinctly by coating type, with calcified stages generally being presented at higher values on the NMDS2 scale within the coating groups (Figure 3.6).




```
Call:
anosim(x = dist, grouping = metadata$Stage)
Dissimilarity: bray

ANOSIM statistic R: 0.1799
      Significance: 0.007

Permutation: free
Number of permutations: 999
```

	1	2	p
1	metamorphosed	settled	0.472
2	metamorphosed	calcified	0.001
3	metamorphosed	cyprid	0.021
4	settled	calcified	0.132
5	settled	cyprid	0.223
6	calcified	cyprid	0.030

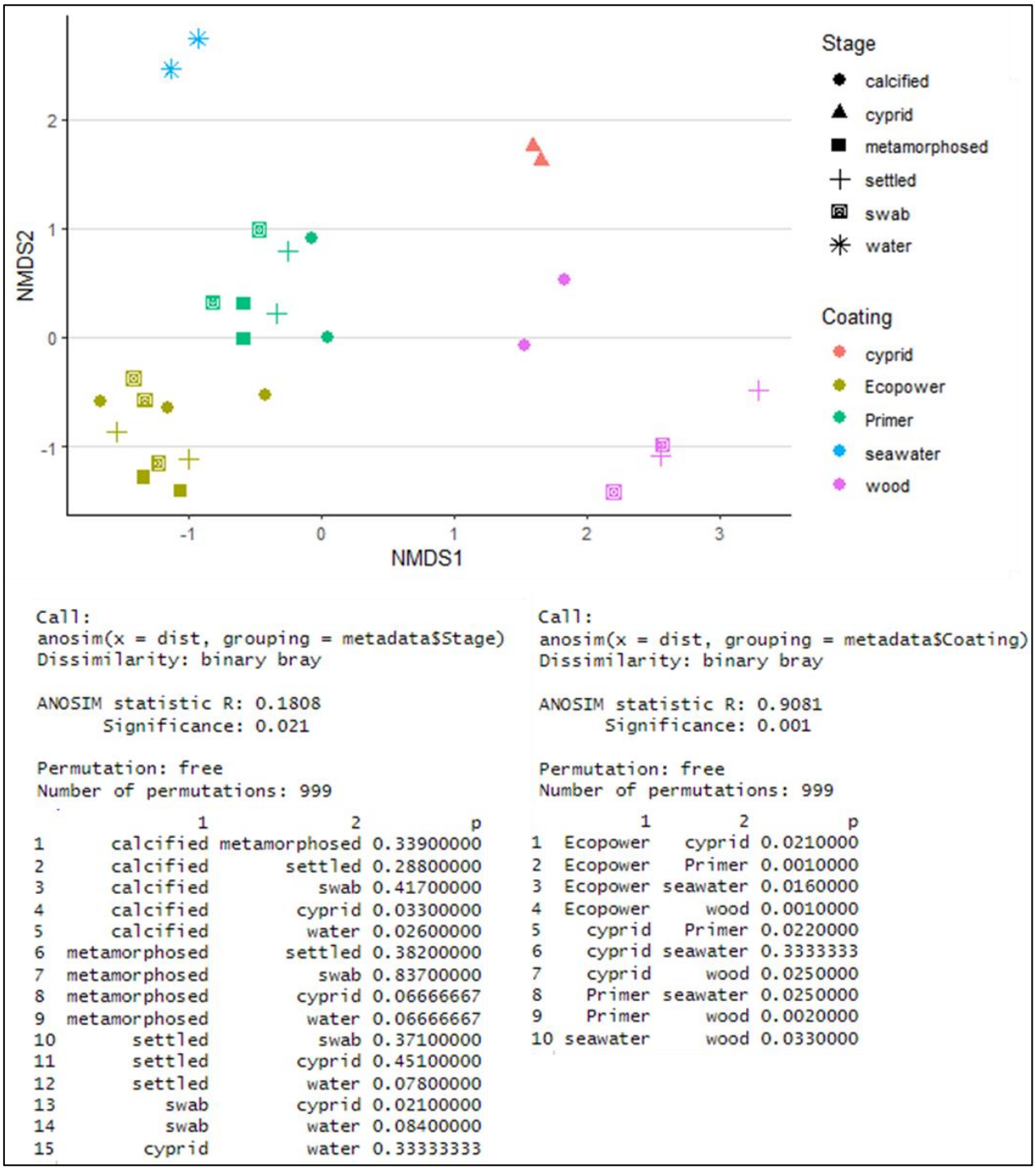
```
Call:
anosim(x = dist, grouping = metadata$Coating)
Dissimilarity: bray

ANOSIM statistic R: 0.8289
      Significance: 0.001

Permutation: free
Number of permutations: 999
```

	1	2	p
1	Coppercoat	Ecopower	0.00300000
2	Coppercoat	cyprid	0.06666667
3	Coppercoat	Primer	0.00600000
4	Coppercoat	Trilux	0.00800000
5	Coppercoat	VCOffshore	0.02800000
6	Coppercoat	wood	0.03500000
7	Ecopower	cyprid	0.02800000
8	Ecopower	Primer	0.00100000
9	Ecopower	Trilux	0.00400000
10	Ecopower	VCOffshore	0.00200000
11	Ecopower	wood	0.00500000
12	cyprid	Primer	0.03200000
13	cyprid	Trilux	0.05400000
14	cyprid	VCOffshore	0.06666667
15	cyprid	wood	0.13333333
16	Primer	Trilux	0.00400000
17	Primer	VCOffshore	0.00600000
18	Primer	wood	0.00600000
19	Trilux	VCOffshore	0.00700000
20	Trilux	wood	0.00900000
21	VCOffshore	wood	0.03100000

Figure 3.5 Beta diversity; left: Bray Curtis PCoA, ANOSIM and pairwise PERMANOVA by settlement stage. Right: Bray Curtis PCoA, ANOSIM and pairwise PERMANOVA by settlement coating type.




```
Call:
anosim(x = dist, grouping = metadata$Stage)
Dissimilarity: binary bray

ANOSIM statistic R: 0.1808
      Significance: 0.021

Permutation: free
Number of permutations: 999
```

	1	2	p
1	calcified	metamorphosed	0.33900000
2	calcified	settled	0.28800000
3	calcified	swab	0.41700000
4	calcified	cyprid	0.03300000
5	calcified	water	0.02600000
6	metamorphosed	settled	0.38200000
7	metamorphosed	swab	0.83700000
8	metamorphosed	cyprid	0.06666667
9	metamorphosed	water	0.06666667
10	settled	swab	0.37100000
11	settled	cyprid	0.45100000
12	settled	water	0.07800000
13	swab	cyprid	0.02100000
14	swab	water	0.08400000
15	cyprid	water	0.33333333

```
Call:
anosim(x = dist, grouping = metadata$Coating)
Dissimilarity: binary bray

ANOSIM statistic R: 0.9081
      Significance: 0.001

Permutation: free
Number of permutations: 999
```

	1	2	p
1	Ecopower	cyprid	0.0210000
2	Ecopower	Primer	0.0010000
3	Ecopower	seawater	0.0160000
4	Ecopower	wood	0.0010000
5	cyprid	Primer	0.0220000
6	cyprid	seawater	0.3333333
7	cyprid	wood	0.0250000
8	Primer	seawater	0.0250000
9	Primer	wood	0.0020000
10	seawater	wood	0.0330000

Figure 3.6 Bray-Curtis dissimilarity NMDS, ANOSIM and pairwise comparisons of all settlement stages on non-biocidal samples only.

3.3.4 Community Composition

Planktonic cyprids were dominated by the following bacterial genera: *Colwellia, Maribacter, Methyloprofundus, Psuedoalteromonas, Rubidomonas, Rubidimionas, Thiothrix,* unclasssifed *Flavobacteriacea* and unclassified *Rhizobiaceae*. Apart from

the Unclassified *Flavobacteriacea* and *Pseudoaltermonas*, the majority of these dominant taxa rapidly decreased across the subsequent life stages (Figure 3.7).

After settlement, the cyprid communities vary in their dominating taxa, depending on what coating they have settled on. Core dominant taxa include: *Colwellia, Cylcoclasticus Spinghorhbadus, Dasania*, unclassified *Flavobacteriaceae*, and unclassified *Rhodobactereacea* (Figure 3.7). The proportion of taxa that make up less than 2.5% relative abundance increased with each development stage, indicating diversification as development progresses.

The community composition of settled samples (settled to calcified) demonstrated coating-specific communities; for example, those on the Ecopower coating were dominated by *Sphingorhabdus* and unclassified *Spongiibacteraaceae*, which were not found in such abundances in the other treatments. The wood communities appeared compositionally distinct from the other samples and were dominated by *Methyloprofudnus, Ilumatobacter, Lewinalla, Phormedismus,* and *Pleurocapsa*, which were not present in such proportions in the other samples. The Coppercoat samples had a higher relative abundance of *Pseudoaltermonas* and *Marinobacter* compared to the other treatments (Figure 3.7).

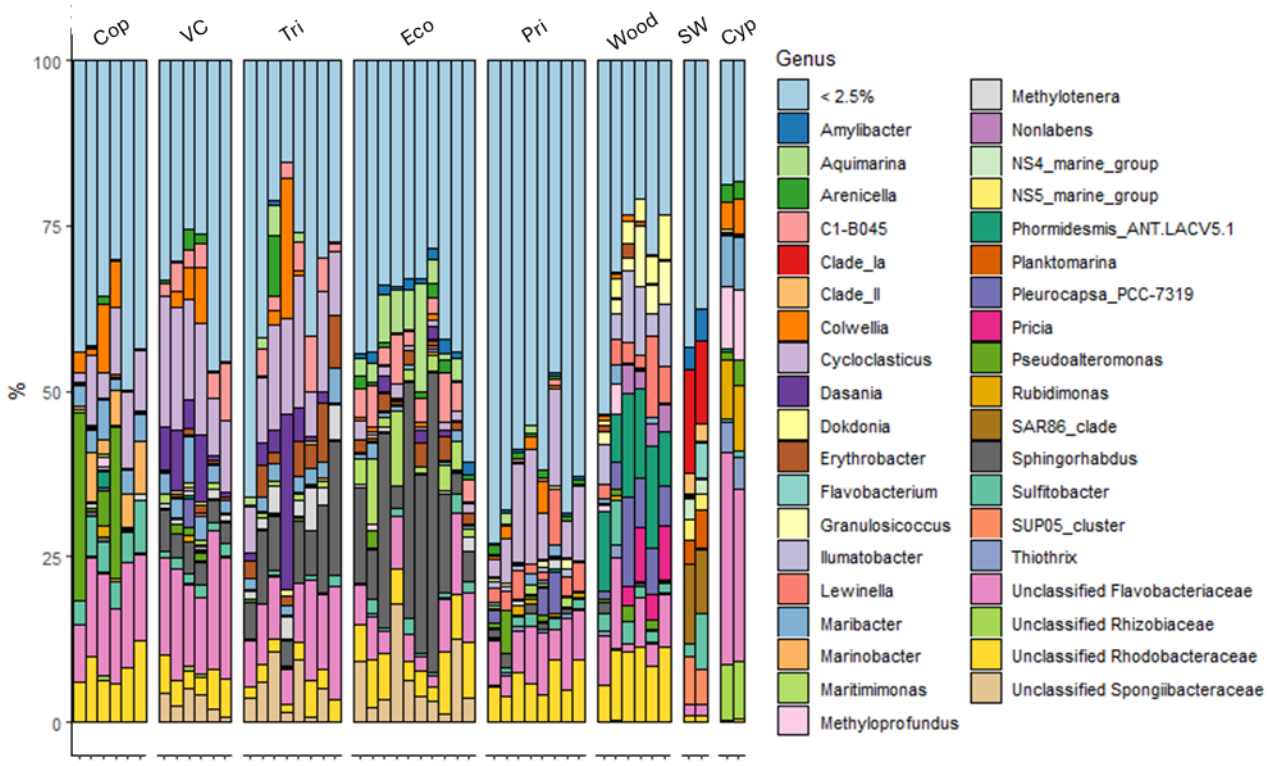


Figure 3.7 Relative abundance of taxa composition at genus level of all samples by coating type. Taxa below 2.5% relative abundance are grouped together in one category.

3.3.4.1 Core Taxa

Most of the taxa identified in this study are found in a small subset of samples (Figure 3.8), indicating variability between samples. In contrast, there are a small number of taxa that are present consistently throughout all life stages and coatings.

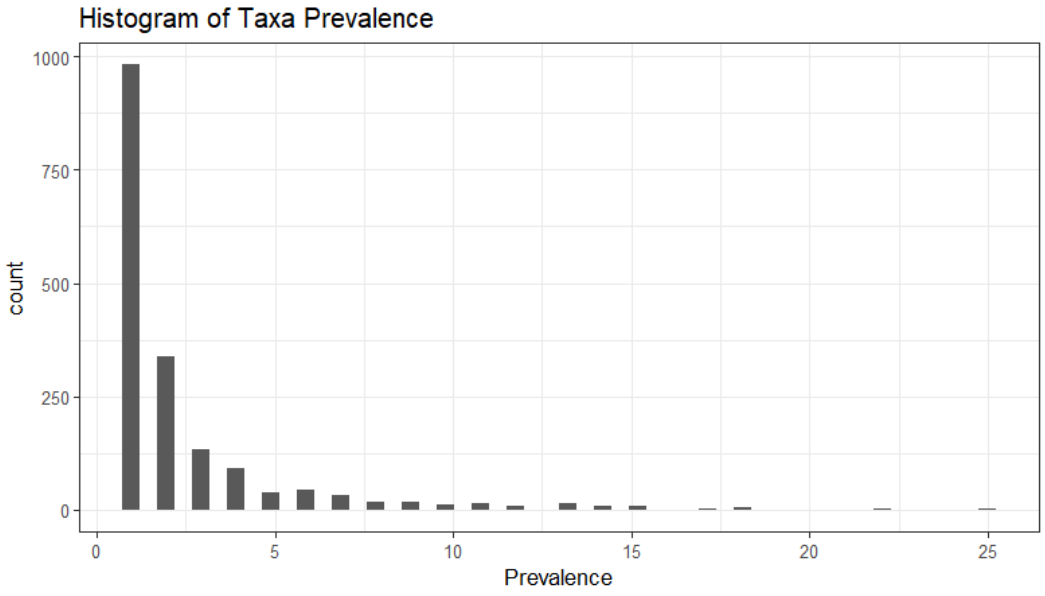


Figure 3.8 Histogram of ASV prevalence across sample numbers.

At a 70% prevalence rate, three taxa were identified: *Methyloligellaceae* sp., *Filomicrobium sp., Alphaproteobacteria* sp. and *Cycloclasticus* sp. These three taxa were not present, however, at the cyprid stage, suggesting that they were acquired at settlement (Figure 3.9). As development progressed, *Cycloclasticus* sp. declined post-calcification.

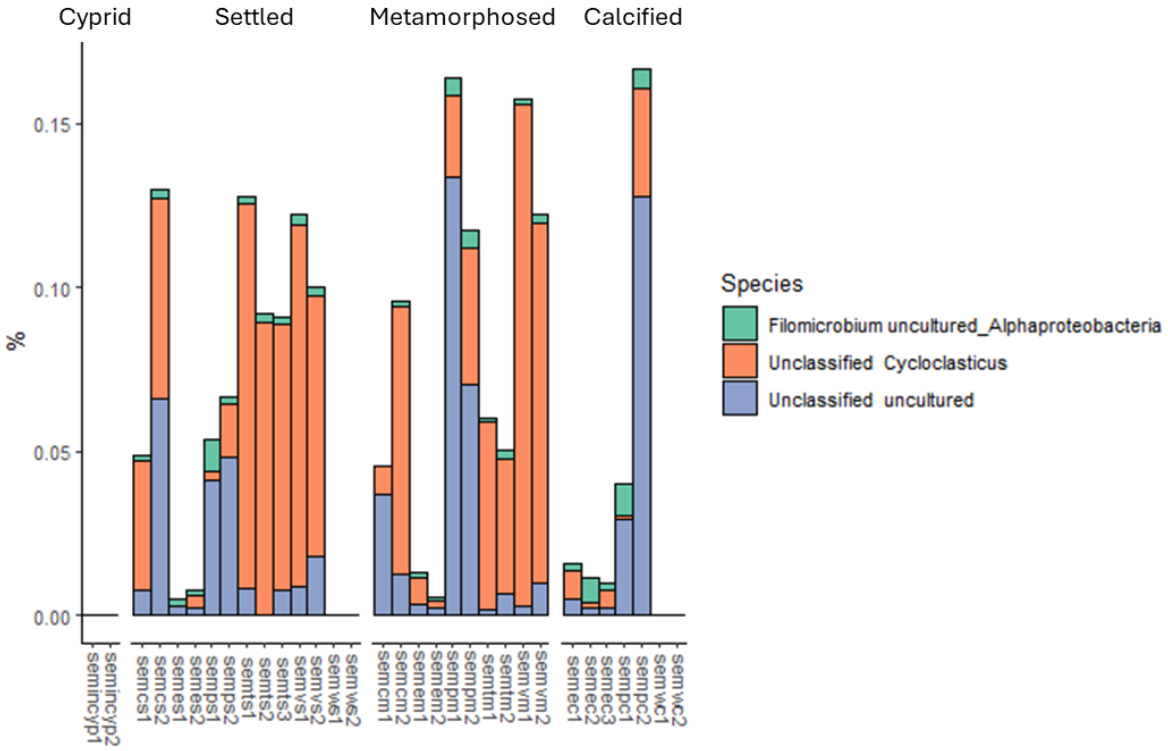
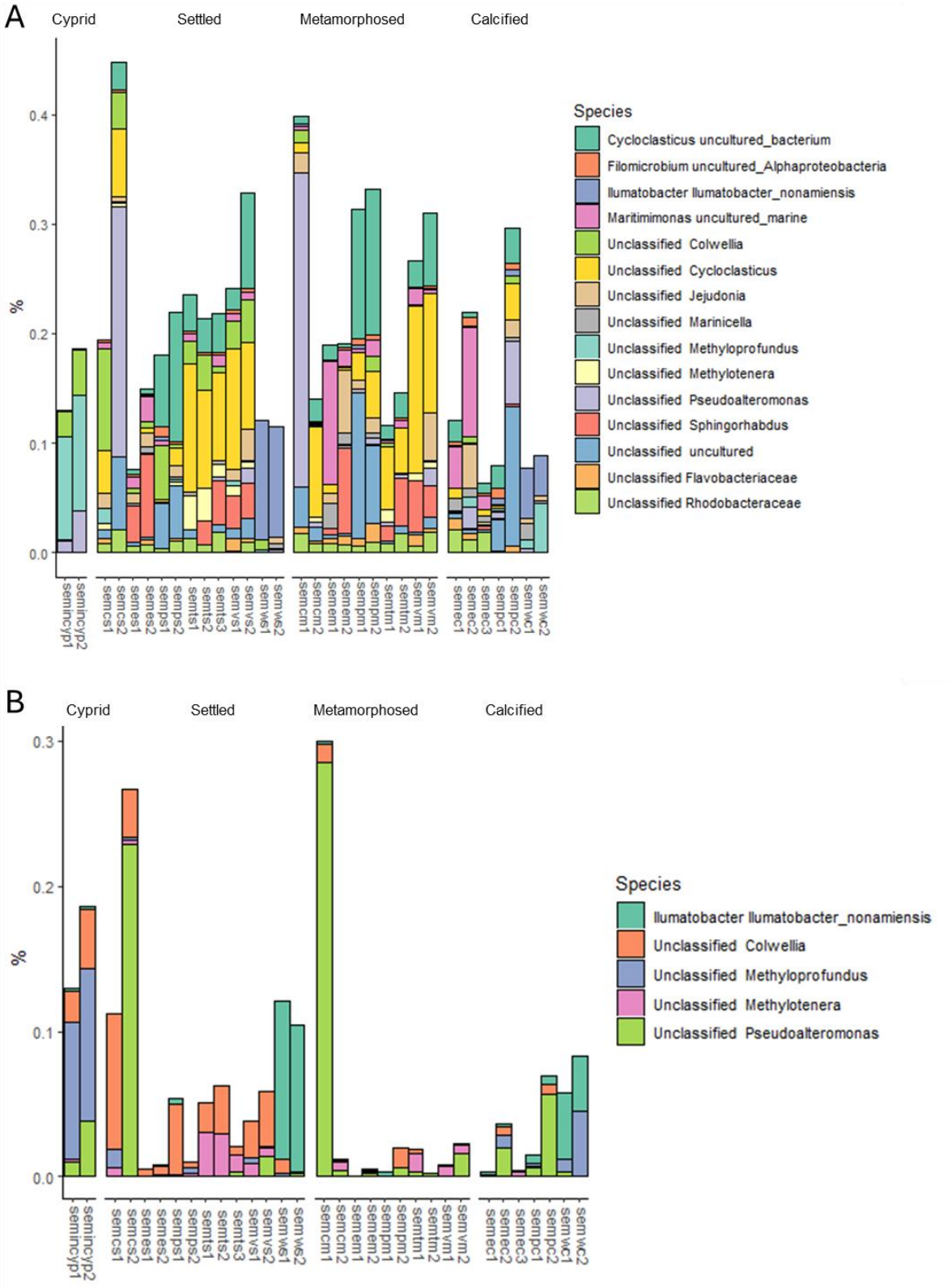


Figure 3.9 Core taxa at a 70% prevalence rate across all Semibalanus balanoides samples. Three taxa were identified: Filomicrobium species, Unclassified uncultured Cycloclasticus and Unclassified uncultured Methyloligellaceae.

At a 50% prevalence rate within *S. balanoides* samples, 18 taxa were identified (Figure 3.10.A): *Sphingorhabdus* sp., two *Rhodobacteraceae* spp., *Methyloligellaceae* sp., *Filomicrobium sp., Alphaproteobacteria* sp., *Maritimimonas* sp., *Flavobacteriaceae* sp., *Jejudonia* sp., *Ilumatobacter nonamiensis*, *Marinicella* sp., *Pseudoalteromonas* sp., *Methylotenera* sp., *Colwellia* sp., four *Cycloclasticus* spp., and *Methyloprofundus* sp.

Of these 18 taxa, five were present at all life stages, from the cyprid to calcified juveniles (Figure 3.10.B): *Ilumatobacter nonamiensis*, *Colwellia* sp, *Methyloprofundus* sp, *Methylotenera* sp. and *Pseudoalteromonas* sp. The contribution to the overall microbiomes of these taxa was relatively small at <0.3%. *Methyloprofundus sp.*, which was found at the cyprid stage, declined in subsequent life stages, apart from those found on the wood samples. *Colwellia* appeared as core taxa throughout all life stages, irrespective of coating. Whereas *Pseudoaltermonas* was lost during settlement on the Ecopower, Primer and Trilux coatings but reappeared after metamorphosis/calcification, suggesting reacquisition.



1714

1715 *Figure 3.10. (A) Core taxa at a 50% prevalence level. (B) Of the 18 core taxa identified,*
1716 *five were present across all life stages.*

1717

3.3.4.2 Differential Abundance

LEfSe analysis identified differentially abundant taxa at each life stage (Figure 3.11, Figure 3.12) and between coatings (Figure 3.13). For life stage comparisons, analysis was done on non-biocidal (Figure 3.11) or biocidal (Figure 3.12) groupings. In the non-biocidal set of samples, there were 24 differentially abundant taxa associated with the planktonic cyprid compared to 18 in the metamorphosed stage. There were only one differentially abundant taxon (Cyanobacteriales sp.) at the settled stage. In contrast, for the biocidal coatings, there were 31 differentially abundant taxa at the planktonic cyprid stage but only six during cyprid settlement and one during metamorphosis (Figure 3.12).

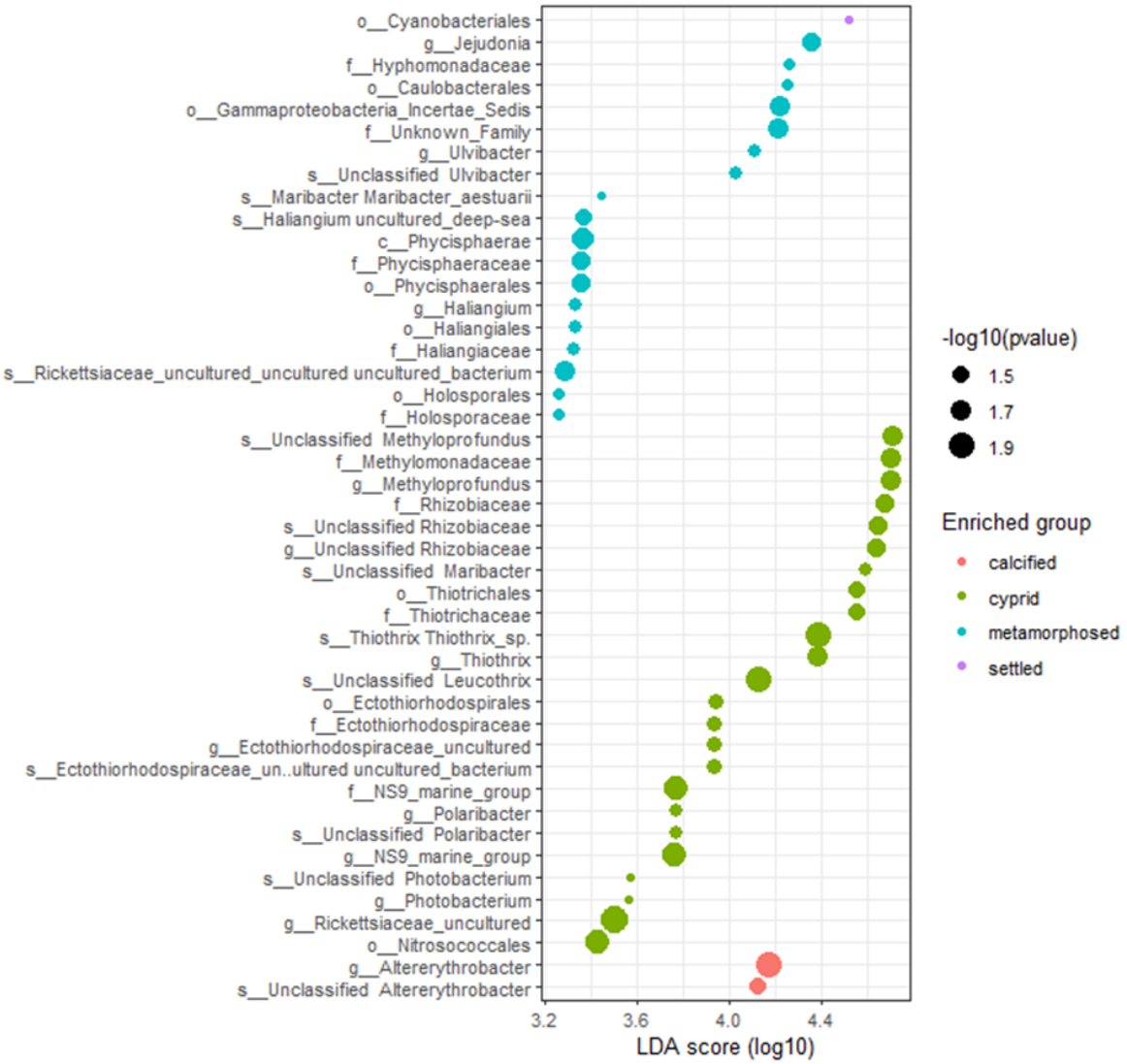


Figure 3.11 LEFSE analysis and log fold change of differentially abundant taxa, colour coded by settlement/development stage of S. balanoides on non-biocidal coatings only. LDA cut off=2. Wilcox and Kruskal Wallis at p=< 0.05. Letters preceding taxa names relate to the relevant lowest taxonomic level achieved.

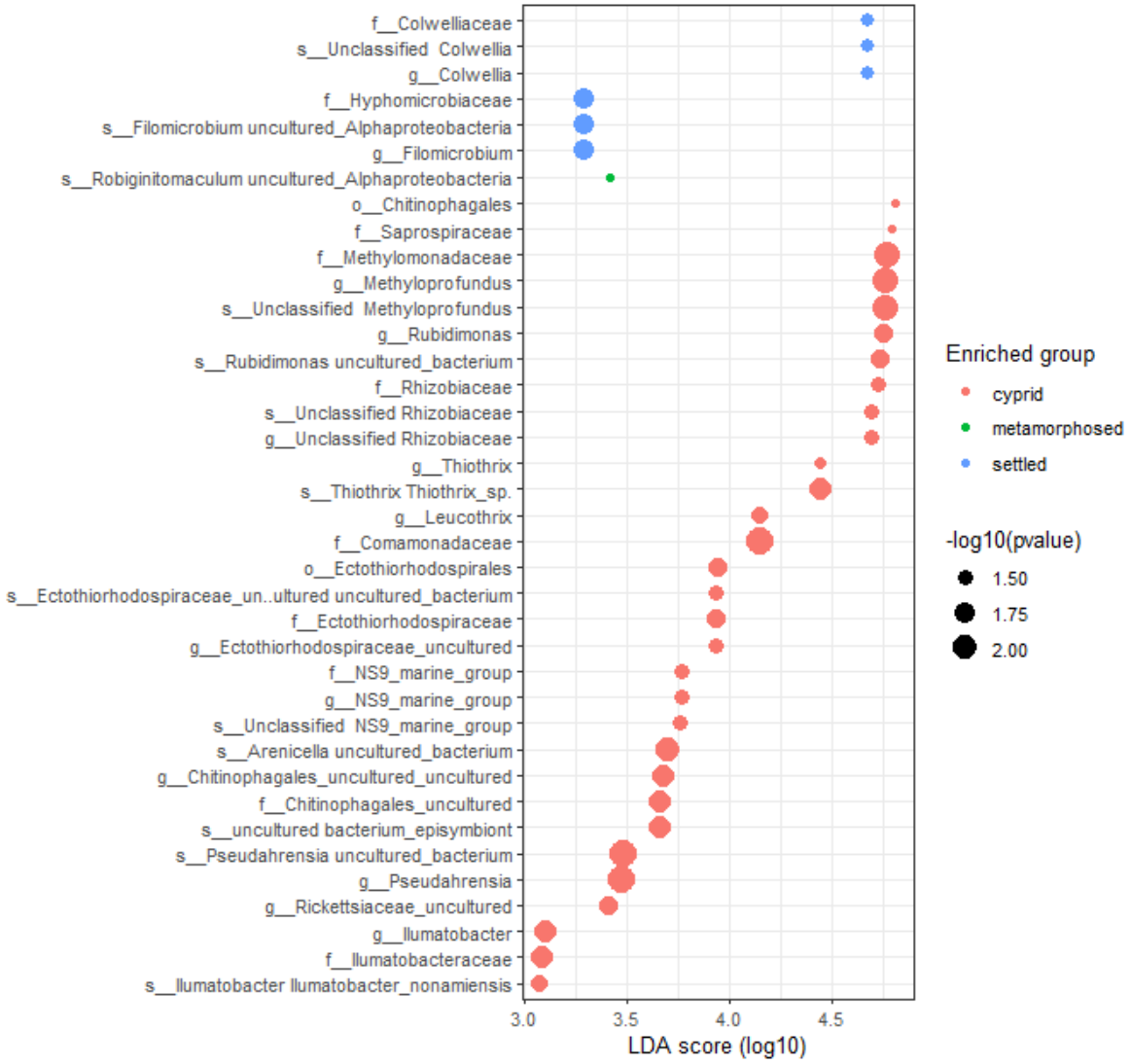


Figure 3.12 LEfSe analysis and log fold change of differentially abundant taxa, colour coded by settlement/development stage of S. balanoides on biocidal coatings only. LDA cut off=2. Wilcox and Kruskal Wallis at p=< 0.05. Letters preceding taxa names relate to the relevant lowest taxonomic level achieved.

When LEfSe analysis was applied by coating, coating-specific taxa were identified (Figure 3.13). The samples from the wooden pier had a high abundance of *Rhodobacterales, Cyanobacteria,* and *Ilumatobacteracea*. VC Offshore had a high abundance of *Ulvibacter* species and *Gammaproteobacteria* strains. Trilux had a high abundance of *Planctomycetes* (OM190), *Gammaproteobacteria* sp, *Proteobacteria* sp, *Cellvibrionales, Spongiibacteracea, Erythorobacter* and *Methylotenera*. Ecopower had a high abundance of *Sphingomondales* sp and *Maritimmonas*. Coppercoat had a high abundance of *Altermondales*, *Sulfiobacter*, *Tenacibaculum* and *Winogradskyella*. The

Primer coating had a larger diverse differentially abundant group, the top three being: *Methyloligellaceae* sp., Plantomycetota sp*. and Lewinalla* sp*.*

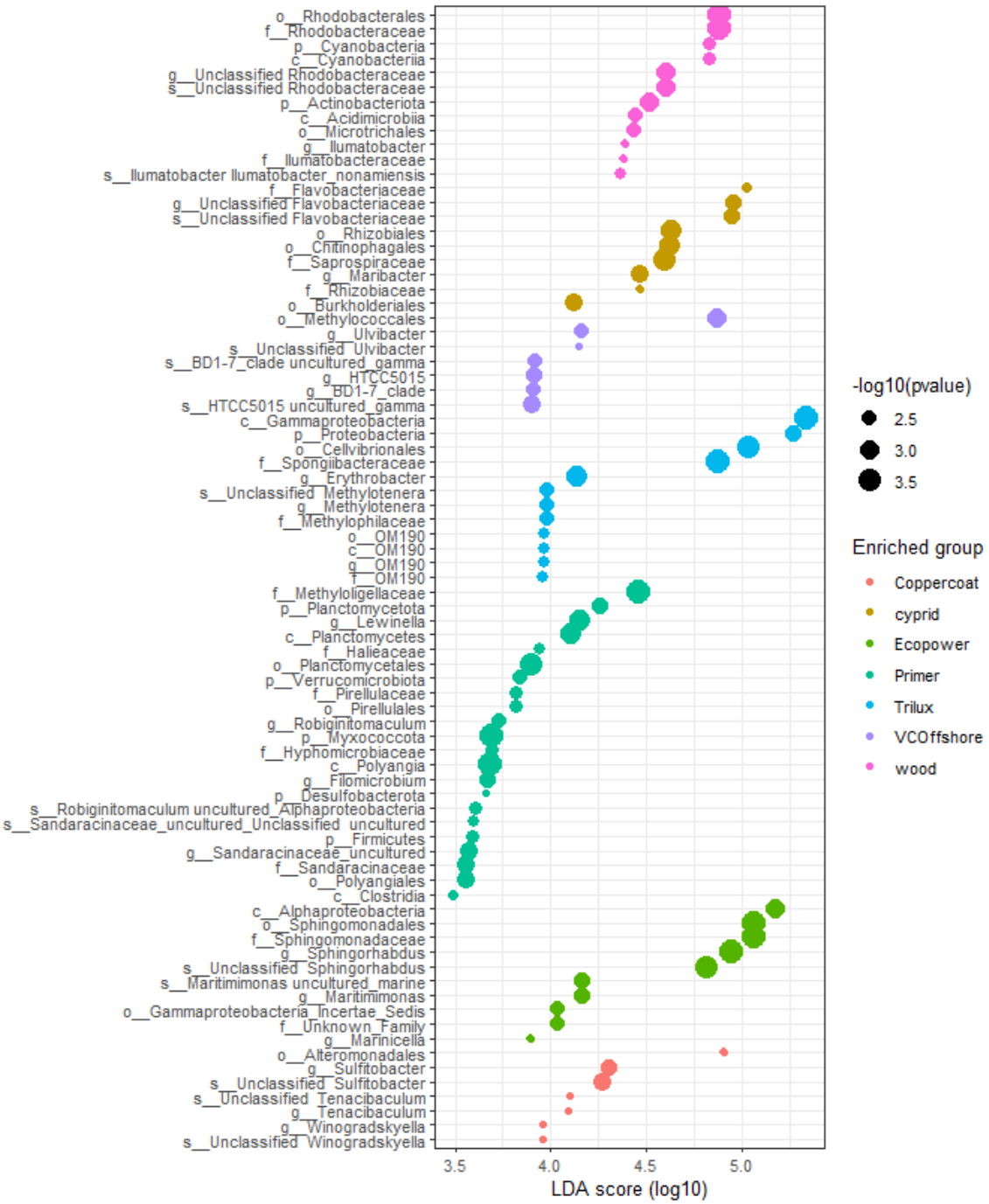


Figure 3.13 LEfSe analysis and log fold change of differentially abundant taxa, colour coded by settlement coating. LDA cut off=2. Wilcox and Kruskal Wallis at p=< 0.01. Letters preceding taxa names relate to the relevant lowest taxonomic level achieved.

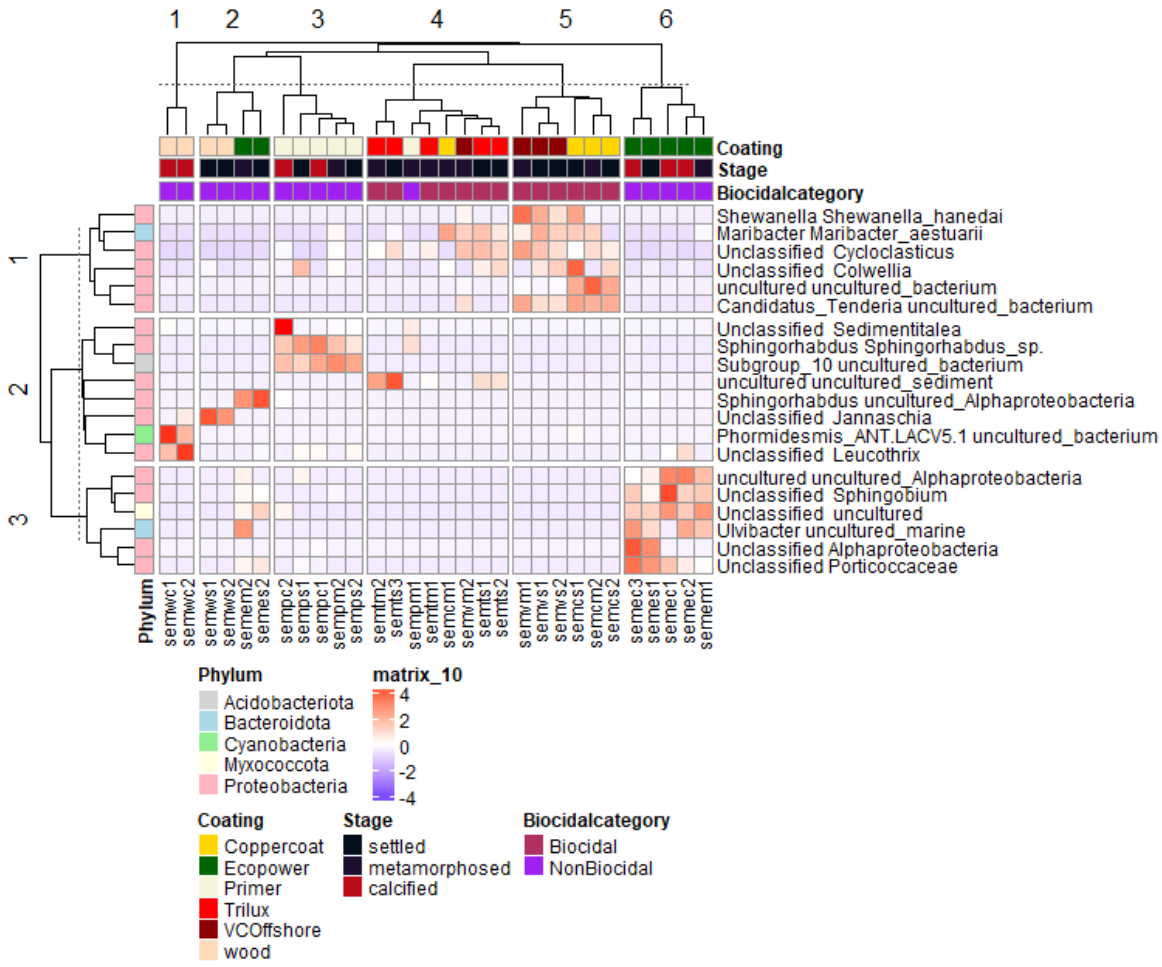


Figure 3.14 Differential abundnace DESEQ2 heatmap grouped by biocidal category.

DESEQ2 identified a selection of differentially abundant taxa based on biocidal vs non-biocidal comparisons. The Ecopower and Primer coatings exhibited the most defined groups of differentially abundant taxa (Figure 3.14) whereas the biocidal coatings showed more overlap. Biocidal coatings are differentially abundant in “group 1” taxa which includes *Shewanella, Maribacter, Cycloclasticus, Colwellia* and *Candidatus Tenderia*. VC Offshore and Coppercoat demonstrated similarly differentially abundant taxa such as *Candidatus Tendaria*. Whereas group 3 taxa featured specifically in the Ecopower coatings and included a few unclassified *Alphaproteobacteria, Sphingobium sp, Ulvibacter sp and Porticoccacae sp.*

3.4 Discussion

This study followed a complementary design to Aldred and Nelson (2019), which looked at the microbiome acquisition of *S. balanoides* during larval settlement. Consistent with the original study, this study has confirmed that the bacteria associated

with the substratum rapidly alter the nature of the barnacle microbiome at settlement, but in this case extended to anti-fouling coatings.

Aldred and Nelson (2019) determined the biggest differences in microbiome shift was between planktonic cyprids and settlement. Similarly, in the present study, planktonic cyprids were compositionally distinct from settled stages (all stages) in beta diversity metrics (Figure 3.5) and community composition (Figure 3.7). The dominant taxa of planktonic cyprids in this study were identified as: *Arencilla, Colwellia, Maribacter, Methyloprofundus, Pseudoalteromonas, Rubidimionas, Thiothrix*, unclassified *Flavobacteriaceae* and unclassified *Rhizobiaceae*. The majority of these taxa rapidly decrease upon settlement, exhibiting drastic community changes and complete shifts towards the new dominant taxa. Although full community data were not available from Aldred and Nelson (2019), they did identify that a *Thiotrichacea* sp*., Thiotrichalea* sp. and *Bizonia sp*. were differentially abundant in the planktonic cyprid samples compared to those that had begun settlement. Although *Bizonia* sp. was not a key species in this study, Thiotrichaceae, specifically *Thiothrix* sp. were found in larger proportions in the cyprid samples compared to those post-settlement (Figure 3.7) and were revealed to be differentially abundant by LEfSe analysis (Figure 3.11, Figure 3.12).

Each barnacle life stage had unique ASVs (Figure 3.3); however there is also a core set of taxa that is present at all life stages (at 50% prevalence rate); from planktonic cyprid to calcified adult (Figure 3.10). Whether these core taxa are carried all the way through their development or reobtained post-settlement is unclear. Taxa such as *Pseudoaltermonas sp.*, appeared to disappear during settlement on the Ecopower, Primer and Trilux coatings, but reappear after metamorphosis/calcification (Figure 3.10). This could be a limitation in sequencing depth and detection, or particular taxa could be reacquired from the environment. Regardless, the coating on which they settle appears to have had a significant effect on the final microbiome composition (Figure 3.7, Figure 3.13, Figure 3.14). Common species across all coating types included: an unclassified *Rhodobacteraceae*, unclassified *Flavobacteriaceae*, *Sulifobactor, Cycloclasticus*, and *Colwellia*. However, there were some species that showed more coating specificity, for example, *Sphingorhabdus* sp. was only found in large proportions on the biocidal VC Offshore, Trilux and the non-biocidal Ecopower coatings. *Dasania* sp. was found on most coatings but in larger proportions on the VC Offshore and Trilux coatings specifically. *Marinobacter* sp., appeared in larger proportions on the Coppercoat coating only, similarly for *Sulfitobacter* and

Pseudoaltermonas, whereas the Ecopower coating demonstrated large relative abundances of *Aquimarina* and *Maritiminmonas*. When compared to the wooden pier, this also had its own unique composition of taxa, dominated by *Phormiodesmis sp., Pleurocapsa sp., Pricia sp., Dokdonia sp., Granulosciocoss sp., Lewinella sp*. Likewise, Aldred and Nelson (2019) identified that *Flavobacteria, Lewinalla, granilosciococis* were differentially abundant in their settled samples on the stone/concrete (uncoated) pier at Cullercoats.

Taxa such as *Candiatis Tenderia* sp. were only found on the Coppercoat and VC Offshore coatings, which both contain copper filings. *C.T. electrophaga* has exclusively been described as “an uncultivated electroautotroph from a biocathode enrichment” (Eddie *et al.*, 2016). The correlation between *Candiatis Tenderia* sp. presence and the copper filings present in the coatings, suggests that the copper may be creating biocathode conditions that is allowing *Candiatis Tenderia* sp. to flourish within these microbiome communities. Further physical characterization of the coatings would be required to confirm this hypothesis.

Shewanella sp., were also found to be differentially abundant on the biocidal treatments (Figure 3.14). *Shewanella* typically consists of anaerobic Gram-negative rods which can be found in extreme aquatic environments (low temperature and high pressure) (Dikow, 2011). *Shewanella* sp. can exhibit stress-resistant characteristics and therefore, may be able to tolerate the stressful environment created by the biocidal coatings, impacting the associated barnacle microbiome, harbouring stress-resistant species. Some *Shewanella* spp. are excellent bioremediators, with versatile metabolic characteristics, including the ability to reduce various electron acceptors (Cai *et al.*, 2022; Darma *et al.*, 2022). They can use toxic substances and heavy metals, which often become less toxic after being reduced (Burns *et al.*, 2010; Luan *et al.*, 2010; Cai *et al.*, 2012), including metals such as zinc and copper (Mugerfeld *et al.*, 2009; Abuladze *et al.*, 2023), which are widely used in anti-fouling coatings.

The LEfSe analysis (Figure 3.13) identified more differentially abundant bacterial taxa per coating compared to the DESeq2 analysis (Figure 3.14). However, the DESeq2 analysis allowed the identification of similar groupings of differentially abundant taxa between biocidal and non-biocidal coatings. This combination of analyses demonstrates that biocidal treatments as a whole have similarities, driving particular taxa (e.g. *Shewanella, Maribacter, Cycloclasticus and Colwellia,* (Figure 3.14)), however, there is also coating-specificity too (Figure 3.13).

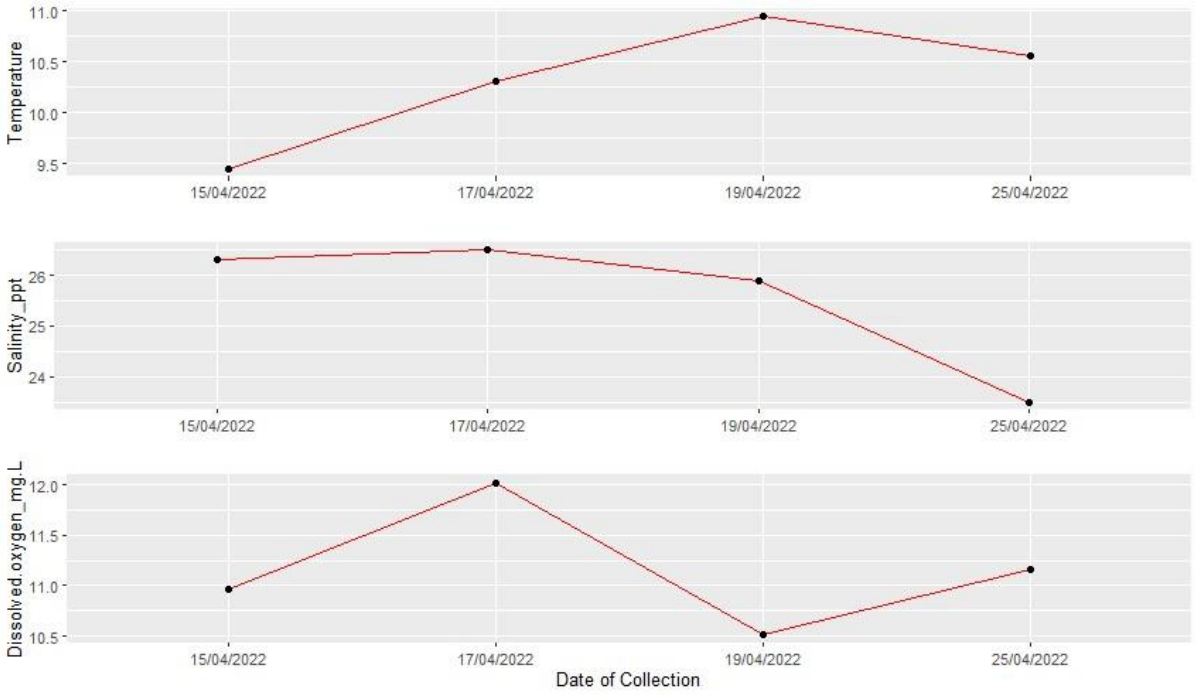
Microbial composition analysis at the calcified stage of *S. balanoides* was only achieved in the non-biocidal treatments in this study, likely due to the poor survival of juvenile barnacles on the biocidal coatings. Several observations were taken during this settlement season: 1) cyprids that settled on the biocidal coatings did so towards the end of the settlement season. 2) Those that did settle on the biocidal coatings were much slower to metamorphose (up to a week post-settlement) compared to those on the non-biocidal coatings where metamorphosis was observed the next day post-settlement. 3) Very few individuals survived to the calcification stage on the biocidal coatings during the sampling period. 4) In contrast, those cyprids that settled on the nearby wooden structures calcified, and rapidly grew in size, a week before those on the treatment panels. It has been established that various barnacle species will search for and inspect suitable substrates before committing to settlement (Knight-Jones and Crisp, 1953; Hills *et al.*, 2000; Prendergast *et al.*, 2008; Maleschlijski *et al.*, 2015). It could be that those that settled on the biocidal coatings did so toward the end of the settlement season and had yet to find a suitable substrate and were therefore 'desperate', settling wherever they could find space before running out of energy reserves (Toonen and Pawlik, 2001; Thiyagarajan, Harder and Qian, 2002; Tremblay *et al.*, 2007). The slower metamorphosis could be due to multiple factors too; 1) delayed settlement with lower cyprid energy reserves having an effect on metamorphosis rates (Thiyagarajan, Harder and Qian, 2002), 2) toxicity of the coatings was preventing/slowing down their development (Rittschof *et al.*, 1992; Cima and Varello, 2022) or 3) the biofilms on the biocidal coatings could have inhibitory effects, as seen with *Amphibalanus reticulatus* larvae, where settlement and metamorphosis was inhibited/prevented on biofilms containing both *Alteromonas* and *Bacillus* spp. (Rajitha, Nancharaiah and Venugopalan, 2020). Although these bacterial species were present in the microbiomes of settled *S. balanoides* in this study, there is no clear relationship identified between these species and the biocidal category (Supplementary Figure 3.4).

The relationship between marine invertebrate settlement and the environmental biofilm is influential and complex. However, the relationships between specific microbial species and barnacle species specifically are understudied. This study provides a starting point in confirming that anti-fouling coating-associated wild biofilms, heavily impact the microbiome acquisition in *S. balanoides*. Although the 'wildness' provides an excellent insight into real world results, the ability to extract species-specific host-

microbe dynamics is not tractable at present under those conditions. This would be a future worthwhile investigation to better understand the relationship of anti-fouling technology and target species.

In summary, the anti-fouling coatings and their associated biofilms lead to differences in *S. balanoides* microbiomes, regardless of the developmental stage, with those on biocidal coatings favouring particular microbial taxa. Changes in community composition to the degree that was found in this study, considering their microbiomes are generally stable across time and space (Aldred and Nelson, 2019; Brown, Nunez and Rand, 2020), warrants further investigation on the impacts this may have on barnacle growth and survival.

3.5 Supplementary

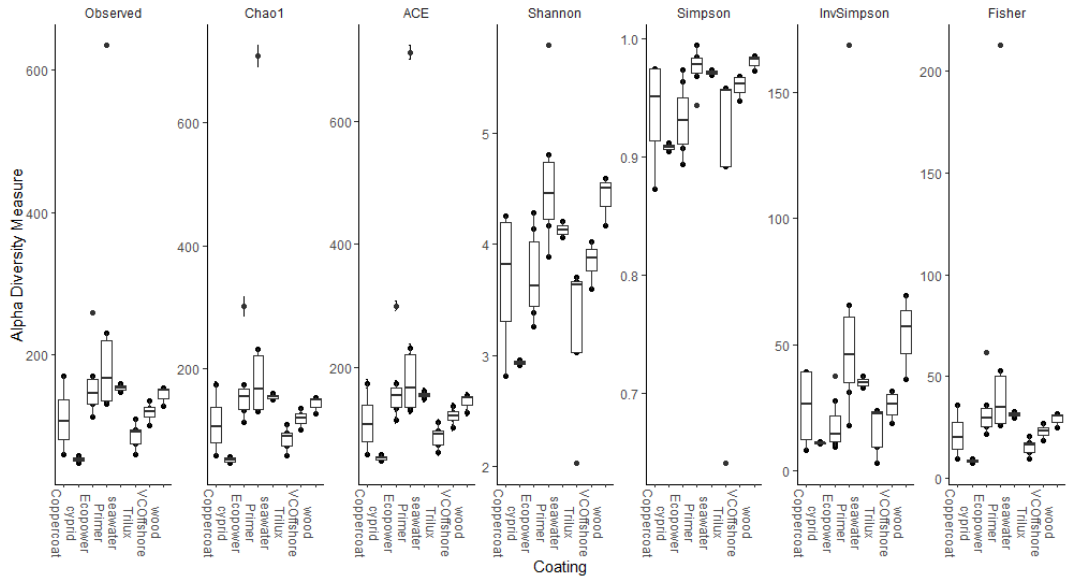


Supplementary Figure 3.1 Environmental measurements on Semibalanus balanoides cyprid settlement collection days at the study site, port of Blyth.

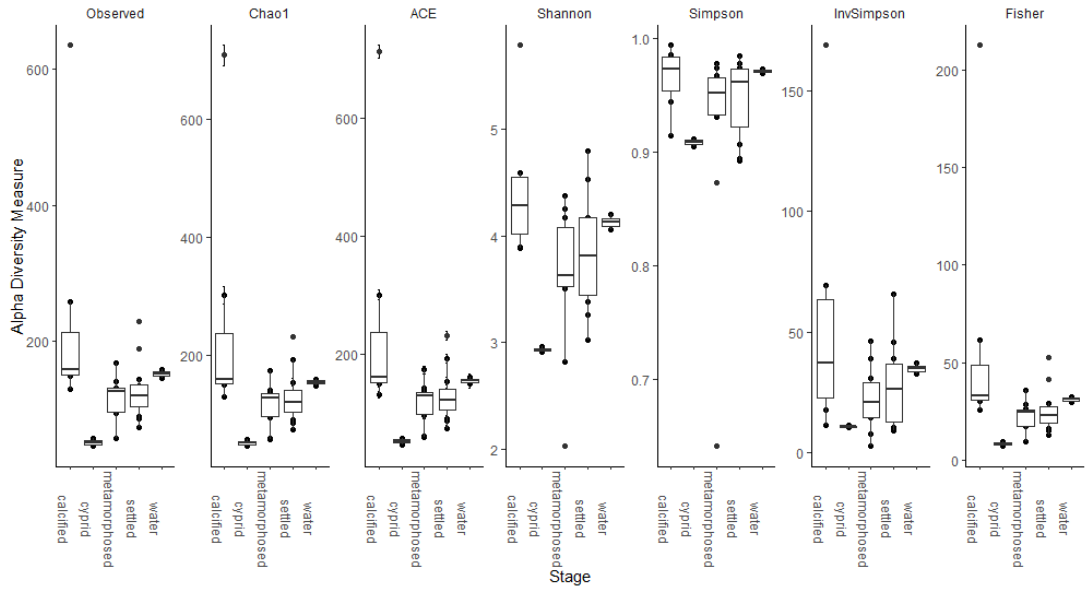
Supplementary Table 3.1 Read counts for each sample post-contamination removal using SCRuB.

semCM1	SemCM2	semcs1	semCS2	semEC1	semEC2	SemEC3	SemEM1
4083	13005	5983	4894	11087	10813	56658	16256
SemEM2	semES1	semES2	SeminCyp1	SeminCyp2	semPC1	semPC2	semPM1
22942	11084	22205	6518	4865	50595	5728	5229
SempM2	SempS1	SempS2	SemTM1	SemTM2	SemTS1	SemTS2	SemTS3
4005	8674	10904	11510	8641	12527	9473	13679
SemVM1	SemVM2	SemVS1	SemVS2	Semwater	Semwater2	SemWC1	SemVC2
11363	9422	13160	8260	14202	12748	7185	7139
Semws1	SemWS2						

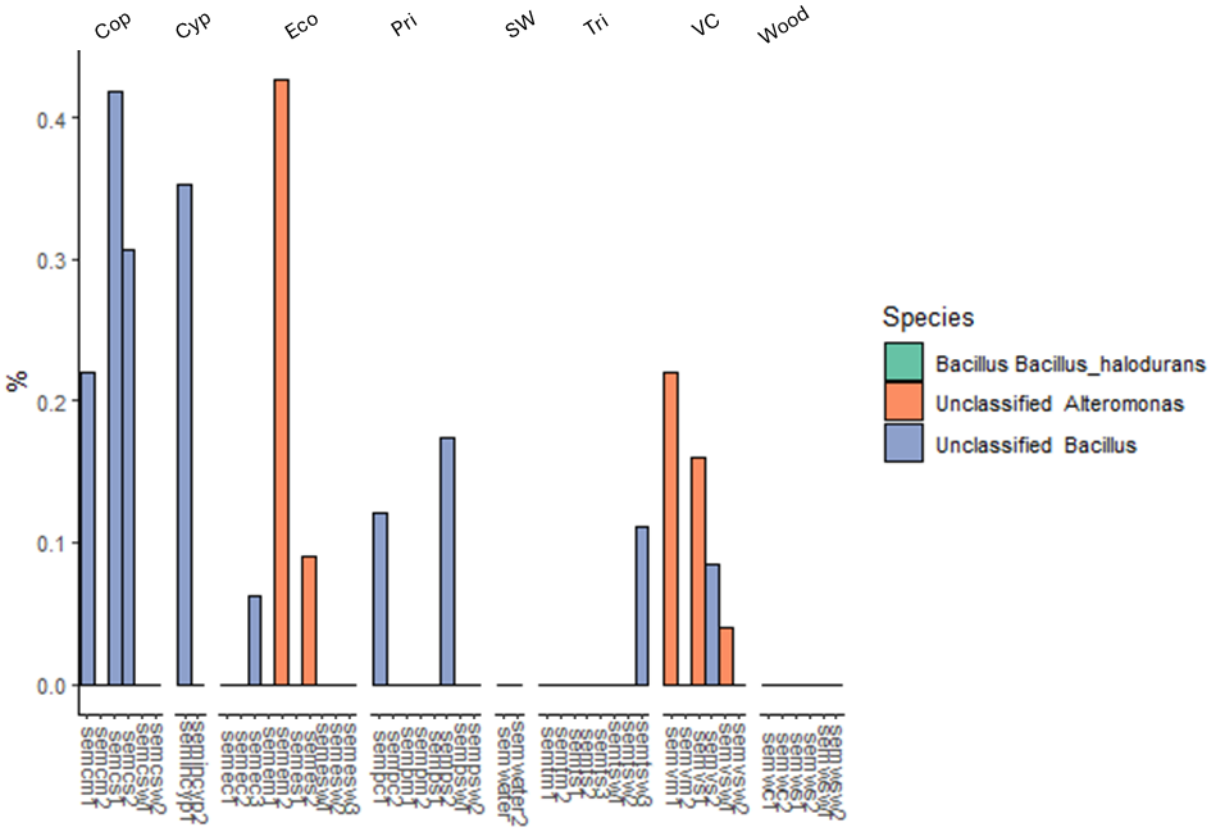
1435	10654						



Supplementary Figure 3.2 Alpha diversity metrics of all Semibalanus balanoides samples by coating type.



Supplementary Figure 3.3 Alpha diversity metrics of Semibalanus balanoides by settlement stage from all coatings.



1895

1896 *Supplementary Figure 3.4 Relative abundance of Alteromonas and Bacillus from*
1897 *Semibalanus balanoides samples during settlement on different anti-fouling coatings.*

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Chapter 4: Fouling Invertebrate-Associated Microbial Communities Change with Host Exposure to Biocidal Coatings

4.1 Introduction

Host-associated microbiomes are complex and diverse, often playing vital roles in the health and ecological functioning of their hosts. These microbiomes are often active participants in the host's life processes, contributing to physiological functions, including digestion, nutrient absorption, and immune defence (Thurber *et al.*, 2009; Wilkins *et al.*, 2019).

One of the primary roles of marine-associated microbiomes is in nutrient cycling and metabolism. For example, in sponges, microbial symbionts are involved in the breakdown of organic matter, which is crucial for nutrient cycling in marine ecosystems (O'Brien *et al.*, 2019). These microbes can fix nitrogen, degrade complex organic compounds and produce essential vitamins and amino acids that the host cannot synthesise on its own (Holt *et al.*, 2022). The next significant role is the microbiome's role in defence mechanisms. Members of the microbiome may help protect their hosts from pathogens through competitive exclusion, production of antimicrobial compounds, and stimulation of the host's immune system (Apprill, 2017; Bowley *et al.*, 2020; Bunker *et al.*, 2021). For example, certain bacteria associated with corals produce antimicrobial compounds that inhibit the growth of harmful pathogens, thereby protecting the coral from diseases (Shnit-Orland and Kushmaro, 2009; Shnit-Orland, Sivan and Kushmaro, 2012; Marangon *et al.*, 2021). Marine invertebrates often live in environments subject to rapid changes and stressors such as temperature fluctuations, pollution, and ocean acidification. The microbiome can help the host adapt to, or mitigate, these changes as evidenced in coral thermal stress experiments (Wilkins *et al.*, 2019). If 'healthy' microbiomes contribute to the resilience of marine communities, they may support the stability and functioning of marine ecosystems (Li *et al.*, 2023).

Despite their importance, marine microbiomes can be disrupted by external influences such as anthropogenic activities (Jurelevicius *et al.*, 2021; Y. Li *et al.*, 2021; Focardi *et al.*, 2022; Kvesić *et al.*, 2022) and environmental change (Thurber *et al.*, 2009; Fan *et al.*, 2013; Y. F. Li *et al.*, 2019; Rubio-Portillo, Esplá and Antón, 2021; Vargas, Leiva and Wörheide, 2021). Pollution and climate change are altering the composition and function of these microbial communities. Shifts in the microbial community structure can result in the proliferation of harmful microbes and the decline of beneficial ones

(Thurber *et al.*, 2009; Quillaguamán *et al.*, 2021; Li *et al.*, 2023). Fortunately, there are some mechanisms that help to reduce the impact of changes to microbiomes and maintain their functional ability (Bell *et al.*, 2018; Swart *et al.*, 2020). Species-specific microbiomes may be conserved between geographies, while also exhibiting local adaptions (Brown, Nunez and Rand, 2020; Utermann, Blümel, *et al.*, 2020; Galià-Camps *et al.*, 2023; López-Legentil *et al.*, 2023).

Because marine invertebrates in biofouling communities can be exposed to a range of environmental conditions, including exposure to biocidal agents within anti-fouling coatings, they have the potential to be prime reservoirs of undesirable microbial species (Flach *et al.*, 2017; Bannister *et al.*, 2019; Imran, Das and Naik, 2019; Destoumieux-Garzón *et al.*, 2020; Georgiades *et al.*, 2021). It is well documented that fouling species can harbour and spread pathogens and disease, impacting ecologically important species as well as commercially important ones, and human health (Waterfield, Wren and Ffrench-Constant, 2004; Fitridge *et al.*, 2012; Costello *et al.*, 2021; Georgiades *et al.*, 2021). Mussels, for example, can accumulate *Vibrio* bacteria, causing gastroenteritis in humans (Destoumieux-Garzón *et al.*, 2020; Sampaio *et al.*, 2022). Tunicate microbiomes can also harbour pathogenic bacteria and fungi, which can cause disease in molluscs (Costello *et al.*, 2021). There is the risk that individuals settled on biocidal anti-fouling coatings may have their microbiomes disrupted or altered, potentially making them more susceptible to pathogens or other undesirable species.

Traditional techniques for bacteria community assessment and pathogen monitoring typically utilise culture-dependant methods (Savichtcheva and Okabe, 2006; Abdelaziz *et al.*, 2017; Plaza-Rodríguez *et al.*, 2021; Murei, Kamika and Momba, 2024). However, marine microbial taxa are diverse and underexplored, and the majority of them have yet to be cultured/deemed culturable. Traditionally, it has been considered only 1% of bacteria from seawater samples can be grown using standard agar plate techniques, although this number has since been disputed, and thought to be much higher (Rodrigues and de Carvalho, 2022). Culture-dependent methods provide valuable insight into pathogen detection and antibiotic resistance profiles, however, will often miss out the large diversity of taxa that are not culturable under standard procedures. Metagenomic techniques have revolutionised bacterial community research, and has allowed the identification of uncultured species and, depending on sequencing depth, the detection of rare taxa and variations/strains, as well as the biodiscovery of new

products and enzymes (Leal *et al.*, 2014; Franklin *et al.*, 2021; Ríos-Castro *et al.*, 2021; Light *et al.*, 2022; Leopold-Messer *et al.*, 2023).

This study aimed to use 16S rRNA metagenomic sequencing to assess whether biocidal anti-fouling coatings impact the microbiomes of fouling invertebrates, focusing specifically on two common invasive/non-native species: *Ciona intestinalis* and *Bugula neritina*. Brief analysis was also conducted on other fouling invertebrates for comparisons: *Austrominius modestus*, *Balanus crenatus*, and *Spirobranchus triqueter*. This study also aimed to determine any differences in whole community antibiotic resistance profiles in biocidal-treated or non-biocidal-treated *Ciona intestinalis* and *Bugula neritina.*

4.2 Methods

4.2.1 Study Site and Sample Collection

The substrate and coatings used in this experiment are the same as those described in Chapters 2 and 3, but they were deployed in Mill Bay Marina, Plymouth, UK (50.363879, -4.152566) (Figure 4.1). Two types of panel were deployed: three matrix boards, as outlined in Chapter 3 (Figure 4.2A), and six sets of individual settlement panels attached to a frame (Figure 4.2B).

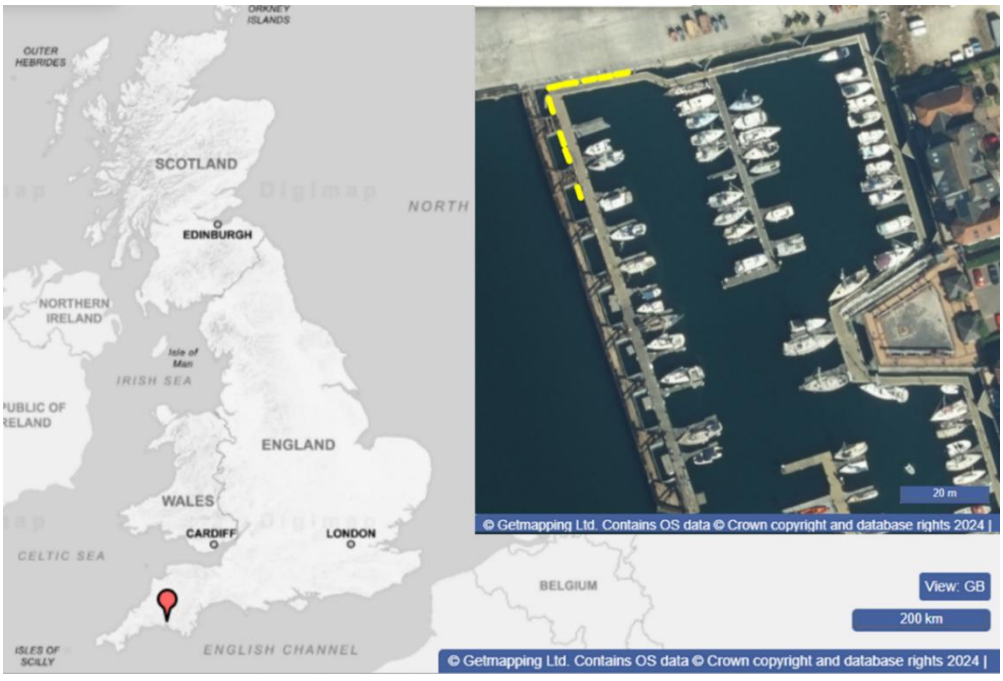


Figure 4.1 Mill Bay study site in Plymouth, UK. The yellow lines represent the locations of settlement panels within the marina. This map was created using © Getmapping Ltd.

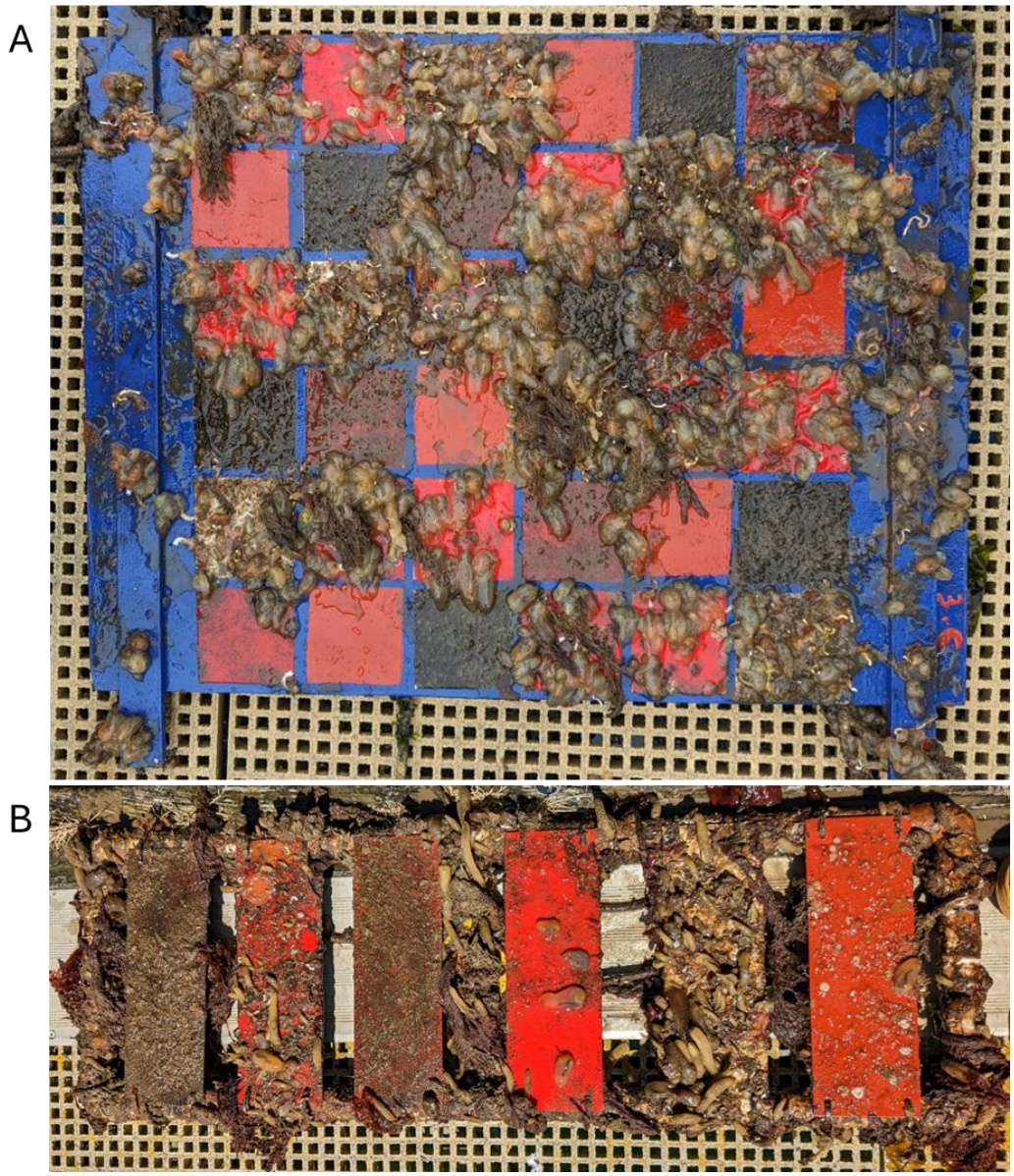


Figure 4.2 Settlement panels during the 2023 settlement season (A) Matrix board with all 6 coatings on, as outlined in Chapter 3. Heavy fouling occurred on the primer, Ecopower and Trilux coatings, primarily dominated by Ciona intestinalis. (B) Individual coating panels attached to a PVC tube frame using the same coatings as the matrix.

Specimens from different taxonomic groups (*Ciona intestinalis, Austrominius modestus*, *Bugula neritina*, *Balanus crenatus*, and *Spirobranchus triqueter*) were removed from replicate biocidal or non-biocidal coatings and placed in sterile 50ml tubes with sterilized natural seawater using sterile forceps (each replicate came from an individual board or panel). The 'bases' of specimens were removed to reduce any contamination from substrate biofilms on the samples. Samples were cleaned within 2-4 hours of collection with a minimum of three washes using sterile seawater, and scraping visible debris using sterile scalpels. Samples were then homogenised within

a microcentrifuge tube and micropestle. Fresh homogenate was used for microbial culture (see section Community Resistance Assays), or frozen at -20°C for DNA extraction (see section DNA Extraction).

4.2.2 DNA Extraction

DNA was extracted from the homogenised samples using two methods: the Powerlyzer Powersoil kit, as per the manufacturer's instructions, for 16S rRNA sequencing and the Zymo Host Zero kit, as per the manufacturer's instructions, for shotgun sequencing. The latter reduced the amount of non-target host DNA (data analysis not included in this thesis).

4.2.3 16S Sequencing and Bioinformatic Analyses

PCR was performed using the Earth Microbiome Project primer set 515F–806R which targets the V4 region of the 16S SSU rRNA (FWD:GTGYCAGCMGCCGCGGTAA (Parada, Needham and Fuhrman, 2016); REV:GGACTACNVGGGTWTCTAAT (Apprill *et al.*, 2015). Using the pre-barcoded primers (Integrated DNA Technology), a PCR was run under the following conditions: 94°C for 3 minutes, followed by 35 cycles of: 94°C for 45s, 50°C for 60s, 72°C for 90s, then a final extension period of 72°C for 10 minutes. PCR was carried out using 0.8x KAPA 2G Robust Taq polymerase (Roche), 0.2µM of each primer and 1µl of DNA. The PCR products were measured using PicoGreen (Invitrogen) on a Spark plate reader (TECAN), then normalised to a concentration of 50nmol/µl. The library was made by pooling 5µl from each sample together prior to clean up using AMPure XP (Beckman Coulter), followed by two washes using 80% ethanol. The library was resuspended in molecular grade water. Library concentration was remeasured using the high sensitivity DNA kit (Invitrogen) using the Qubit 4.0 (Invitrogen) and the product size was checked using the high sensitivity DNA kit (Agilent) on the Agilent Bioanalyzer 2100. The library was dissociated into single strand DNA using an equal volume of 0.2N sodium hydroxide which was stopped after 5 minutes using 400nM Tris pH 8.0 buffer. The library was then diluted to 20pMol with Illumina hybridisation buffer before being spiked with 20% v/v PhiX (phage lamda DNA) as in internal quality control. The final library was loaded into the Illumina MiSeq v2 500 cycle reagent cartridges.

Quality-filtered, demultiplexed fastq files were processed using Qiime2-2023.2 (Bolyen *et al.*, 2019). Sequences were quality trimmed based on the 25th percentile at a minimum of 30 x quality score (forward 243, reverse 14 to 208). Samples were denoised using DADA2 (Callahan *et al.*, 2016), producing unique sequences (5598

features over 65 samples, 1136206 total reads). Chimeras were identified and removed using UCHIME (Edgar *et al.*, 2011), along with very low abundant features (less than 10). Sequences were aligned to the v4 region of the 16S rRNA gene sequences from the SILVA v138 database (Quast *et al.*, 2013). Non-desirable sequences were removed that were classified as eukaryote, mitochondria, archaea, chloroplast or unknown according to SILVA taxonomies. A Phylogenetic tree was built using Fast-Tree. Feature tables, taxonomies, rooted tree and metadata were exported for downstream analysis in RStudio (R Core Team, 2023). Predicted functional KEGG orthologs were created using the PICRUSt2 pipeline (Douglas *et al.*, 2020).

4.2.3.1 Data Visualisation and Statistical Analysis

Potential contamination identified in the negative controls was removed using SCRuB (Austin *et al.*, 2023). Rarefaction, diversity metrics and community composition were conducted using Phyloseq (McMurdie and Holmes, 2013) and Vegan (Oksanen *et al.*, 2024) packages. Mixed fouling invertebrate data were rarefied to 4679 to include as many samples as possible. For species-specific analysis, *C. intestinalis* data were rarefied to 8387, and *B. neritina* data was rarefied to 8510. Beta diversity and associated distance plots were conducted through Phyloseq and ggplot2. Differential analysis was conducted using DESEQ2 (Love, Huber and Anders, 2014). Pairwise comparisons of phyla were conducted using Wilcoxon tests. PICRUSt2-predicted KEGG functional comparisons were visualised using STAMP (Parks *et al.*, 2014), and beta diversity of KEGGs visualised using ggplot2 in R.

4.2.4 Community Resistance Assays

Samples were homogenised in 500µl of marine broth (Millipore), then diluted to 1 in10, 1 in 100, 1 in 1000 or 1 in 10000 depending on the amount of starting material. Fifty µl was added to pre-treated antibiotic marine agar (1.5%) plates (Table 4.1 for concentrations) and control plates with no antibiotics. All plates were incubated for 24 hours at 18^{o}C before counting. Counts from amended plates were divided by the counts from non-amended plates to calculate the proportion of resistant bacteria. Three biological replicates per antibiotic per species were conducted. Only counts from the dilution plates that ranged from 30-300 colonies per plate were used.

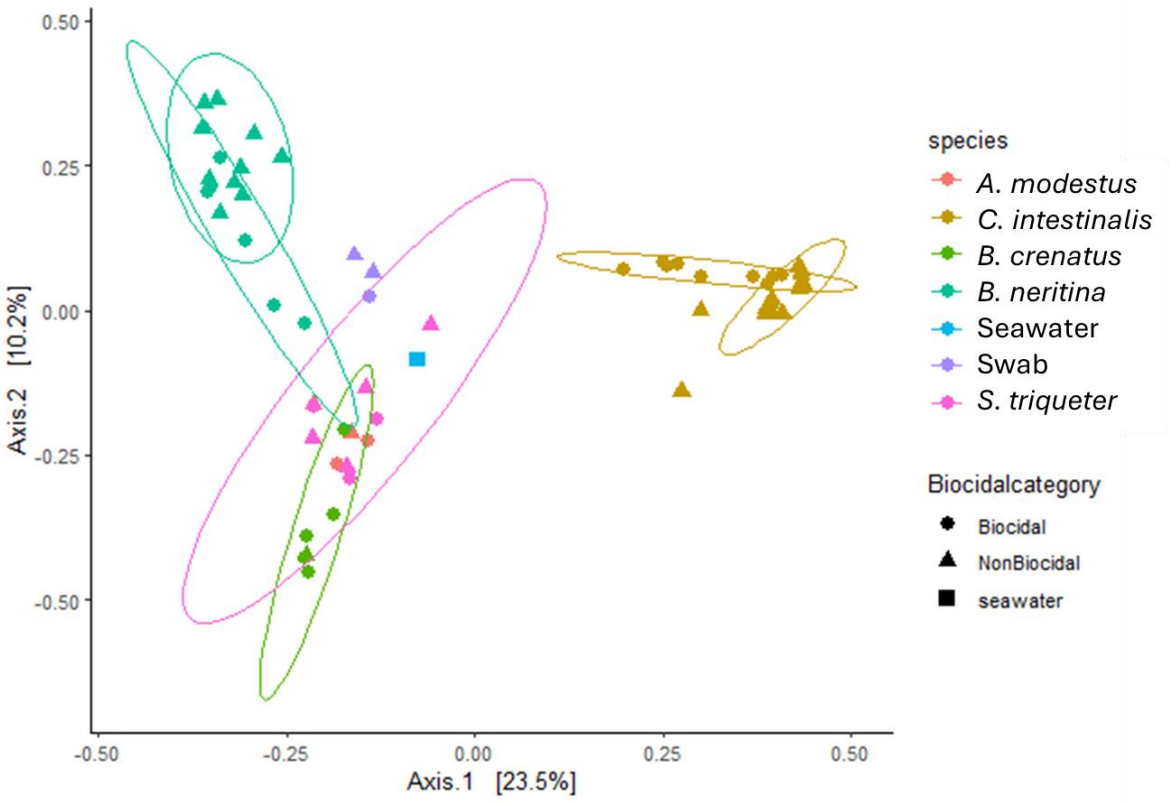
Table 4.1 Antibiotic concentrations for whole community resistance assays. Concentrations based on average EUCAST MICs for Vibrio spp. and Pseudomonas spp.

Antibiotic	Concentration	Type
Tetracycline	2mg/l	Tetracycline
Kanamycin	12mg/ml	Aminoglycoside
Ampicillin Trihydrate	8mg/l	Beta-lactam
Gentamicin	4mg/l	Aminoglycoside
Ciprofloxacin	0.5mg/l	Quinolone

4.3 Results

4.3.1 Mixed Fouling Species Microbiome Characteristics

Each species had its own distinct microbial community association based on beta diversity metrics (Bray-Curtis dissimilarity) (Figure 4.3). Even with species-specific variability, there was a diversity shift between biocidal and non-biocidal micorobiomes as seen in the *C. intestinalis* and *B. neritina* associated communities (Figure 4.3). Alpha diversity between biocidal and non-biocidal treatments varies between fouling species (Figure 4.4). Microbial diversity was higher on the non-biocidal coatings for *Austrominius modestus* and *Bugula neritina*, however, diversity was either the same or higher on the biocidal coatings for the other species (*Ciona intestinalis, Balanus crenatus*, and *Spirobranchus triqueter*) (Figure 4.4).



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2085 *Figure 4.3 Bray-Curtis PCoA plot of all species microbiome samples collected in this*
2086 *study. C. intestinalis and B. neritina communities illustrate differences in beta diversity*
2087 *based on the biocidal group it was exposed to.*

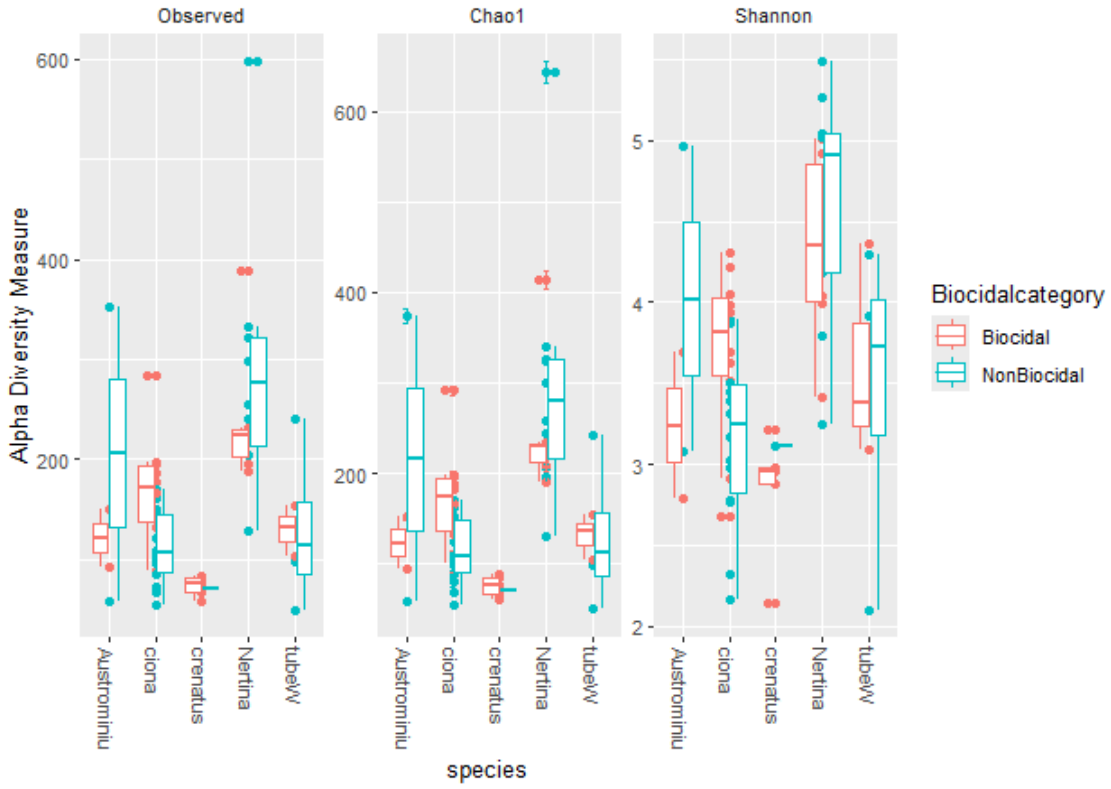


Figure 4.4 Alpha diversity metrics across the different fouling species on biocidal and non-biocidal coatings. Red= Biocidal. Blue= Non-biocidal.

Species specific community composition was examined at the order taxonomic level (Figure 4.5). Although *A. modestus* and *B. crenatus* (both barnacle species) displayed some overlap in relation to beta diversity (Figure 4.3), the overall composition was compositionally distinct between the two species. *A. modestus*' dominant taxa were Sphingomondales, Rhodobacterales and Cellvibrionales, whereas the dominant taxa associated with *B. crenatus* were Vibronales, Rhodobacterales and Flavobacteriales. In *A. modestus* samples, Sphingomondales had a higher relative abundance from the biocidal Trilux coating and ~50% proportionally fewer Rhodobacterales were present. Similarly, *C. intestinalis* Trilux and VC Offshore samples, had a considerable reduction in Oceanospiralles, but a major increase in Pirellulales. In *B. neritina* samples from the primer coatings, there were marginally fewer Alteromandales, but otherwise there were similar proportions of dominant taxa at the order level across the coatings.

B. crenatus from the Coppercoat coating lacked Vibronales compared to the other coatings. *S. triqueter* samples from the Trilux coatings typically had larger relative abundances of Rhodobacterales compared to the other coatings.

Figure 4.5 Order level relative abundance community composition by species. those that had relative abundances below 2.5% were grouped together as '<2.5%'. coating labels are: eco=ECOPOWER, tri=TRILUX, cop=Coppercoat, pla=Plastic, pri=Primer, sil=SilicOne, vc=VC Offshore, sw = seawater. The number following the coating label refers to replicate number.

Full community data for all invertebrate species microbiomes were processed through PICRUSt2 to predict KEGG ortholog differences between biocidal and non-biocidal treatments (Figure 4.6). Biocidal treatments showed an abundance of KEGGs related to manganese/zinc/iron transport systems, while non-biocidal treatments showed an abundance of KEGGs related to V/A-type H+/Na+ transporting ATPase subunits.

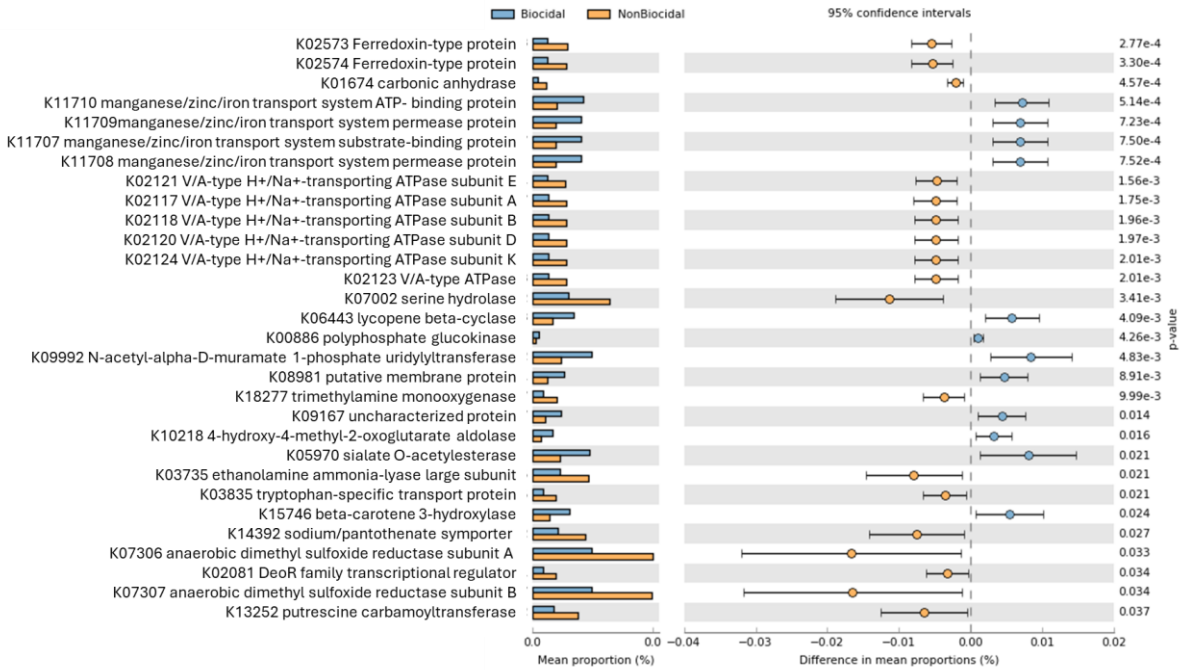


Figure 4.6 PICRUSt2 STAMP analysis comparing all species, biocidal vs non-biocidal KEGG orthologs. Blue: Biocidal. Orange: Non-biocidal.

Due to issues obtaining replicates from all biocidal coatings across all species, further analyses focused on the microbiomes of *Ciona intestinalis and Bugula neritina* only.

4.3.2 *Ciona intestinalis* Microbiome

Relative community composition revealed that *C. intestinalis* was dominated by 12 taxa that contribute to more than 2% of the overall community (Figure 4.7). Of those dominant taxa, there was variation in the quantities across the coating treatments. Samples from *C. intestinalis* from VC Offshore and Trilux coatings had a significant reduction in the relative abundance of *Endozoicomona*s compared to other treatments. Biocidal coatings, in general, have less 'unclassified Alphaproteobacteria'. Although *Vibrio spp.* is present across all treatments, there are larger relative abundances within the non-biocidal treatments. VC Offshore and Trilux samples had a higher proportion of species below 2% relative abundance, suggesting there are more species but with smaller community contributions.

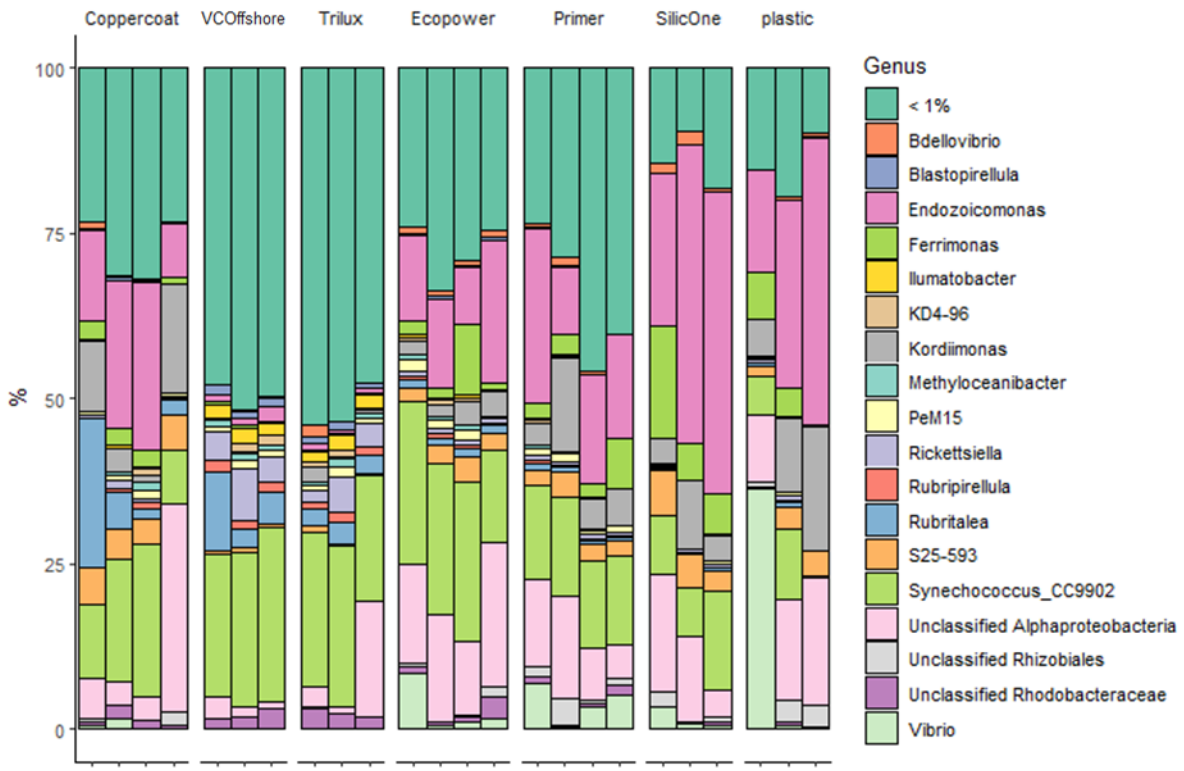


Figure 4.7 Genus level taxonomic profile of C. intestinalis associated microbiomes from different anti-fouling coatings.

At the phylum level, there were nine significant differences detected between the biocidal and non-biocidal grouped relative abundances (Figure 4.8). Non-biocidal treatments had more Dellovibrionota and Proteobacteria, whereas the biocidal treatments had more Acidobacteriota, Actinobacteriota, Euryarcheota, Verrucomicrobiota, Dependentiae, Gemmatimonadota and Planctomycetota. The relative abundance differences found within the biocidal treatment appeared to be driven by the 'mixed biocidal' coatings, Trilux and VC Offshore, while Coppercoat displayed similar abundances to the non-biocidal treatments (Supplementary Figure 4.1).

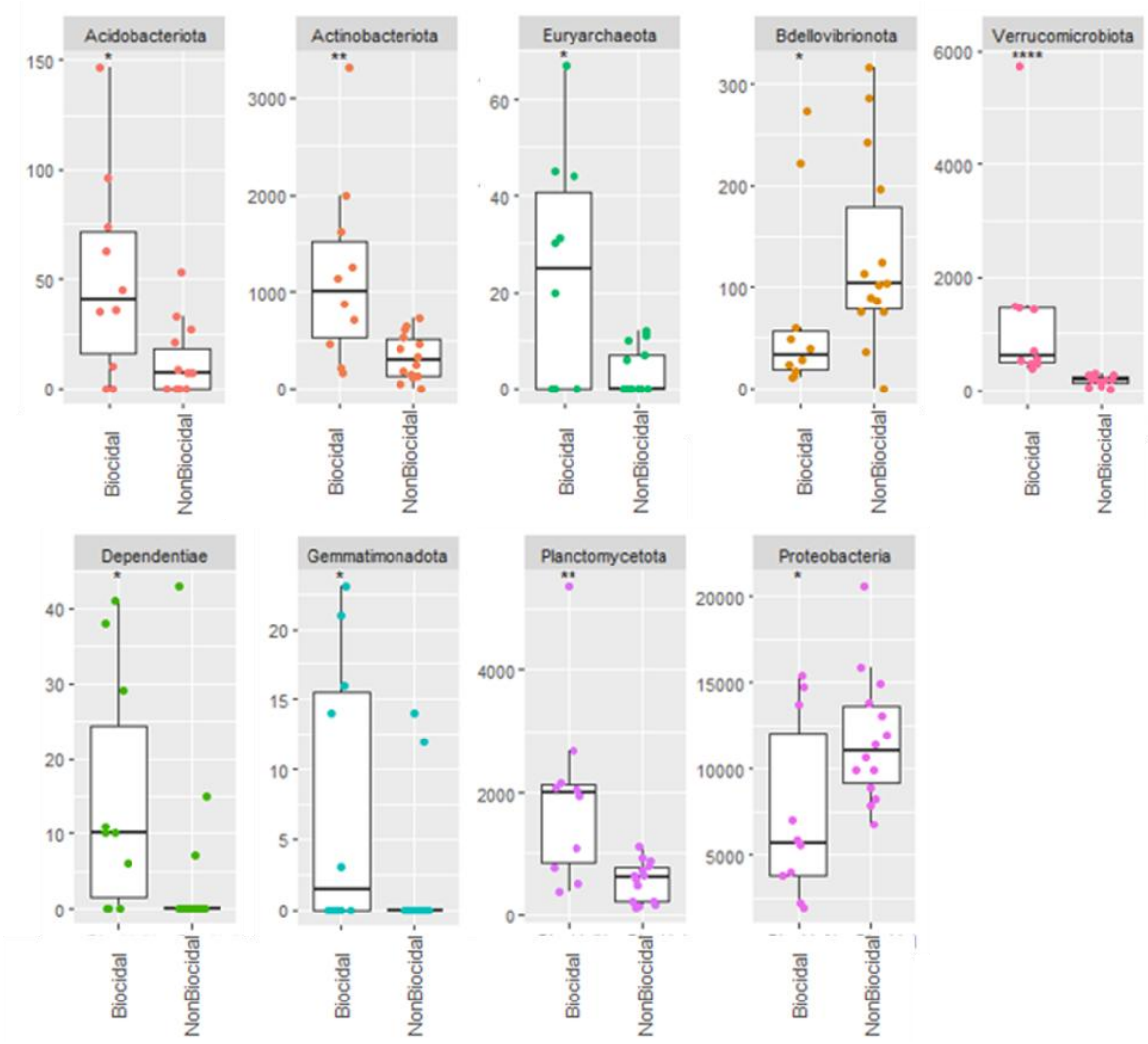


*Figure 4.8 Phylum level biocidal vs non-biocidal mean relative abundance boxplots with Wilcoxon statistical comparisons from C. intestinalis microbiomes. *:p<=0.05, **:p=<0.01, ****:p<0.0001. For full phyla-level comparisons by coating see Supplementary Figure 4.1.*

Using DESeq2 differential analysis and clustered dendrogram, the biocidal treatments grouped together for groups 1 and 2 (Figure 4.9), demonstrating similar abundant taxa within each grouping. Group 1 consists of Trilux and VC Offshore coatings, which had an abundance of *Windogradskyella* sp., *Candidatus_Navis* sp., *Amylibacter* sp., *Neptuniibacter* sp. and *Pontibacterium* sp. Group 2 consists of the Coppercoat treatment with one Ecopower sample, however with no obvious differentially abundant taxa.

Groups 3 and 4 (Figure 4.9) represent the remaining non-biocidal treatments, with a mix of Ecopower and Primer coatings. Group 3 is dominated by the primer coating, and Group 4 is dominated by the Ecopower coating. Differentially abundant taxa in

group 3 consist of: *Vicingus* sp., *Fabibacter* sp., *Woesiea* sp., uncultured gamma sp., *Candiatus_amoaebophilus* sp., *Candiatus_Endobugula*_endosymbiont sp. Group 4 differentially abundant taxa include EC3 uncultured bacterium. *Candiatus_Amoebophilus* sp., *Litoreibacter* sp., *Sulfurovum* sp., *Beijerinckiacea* sp., *Pirellula* sp., Acidobacteriacea sp., uncultured bacterium sp., and a Sva0996 marine uncultured sp.

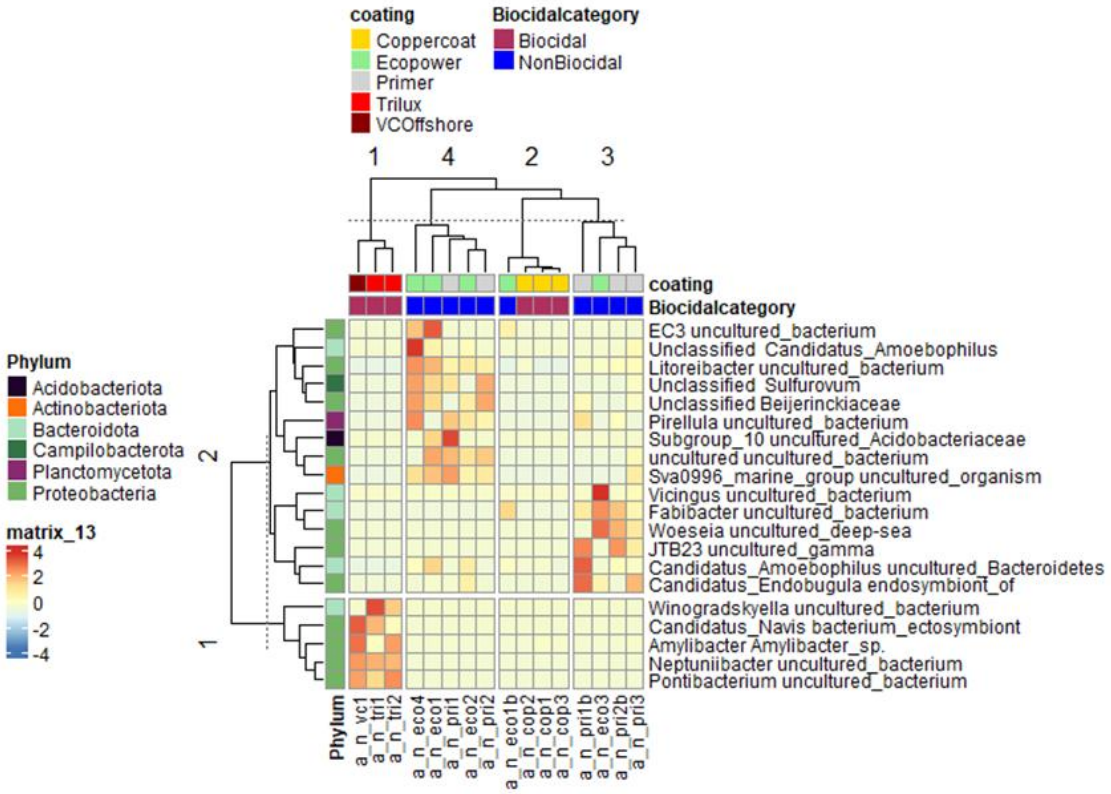


Figure 4.9 DESeq2 heatmap analysis of C. intestinalis microbiomes between coatings in the biocidal and non-biocidal treatments.

Bray-Curtis dissimilarity PCoA illustrated differences in the community composition between the biocidal and non-biocidal groups. Although there was overlap, the differences between the biocidal groups were significant (ANOSIM, R=0.4907, *p*=0.001) (Figure 4.10). Using predicted functionality based on community compositions, there was a differential shift in the KEGG orthologs between biocidal and non-biocidal coatings (Figure 4.11A), which were driven by the Coppercoat samples (Figure 4.11B).

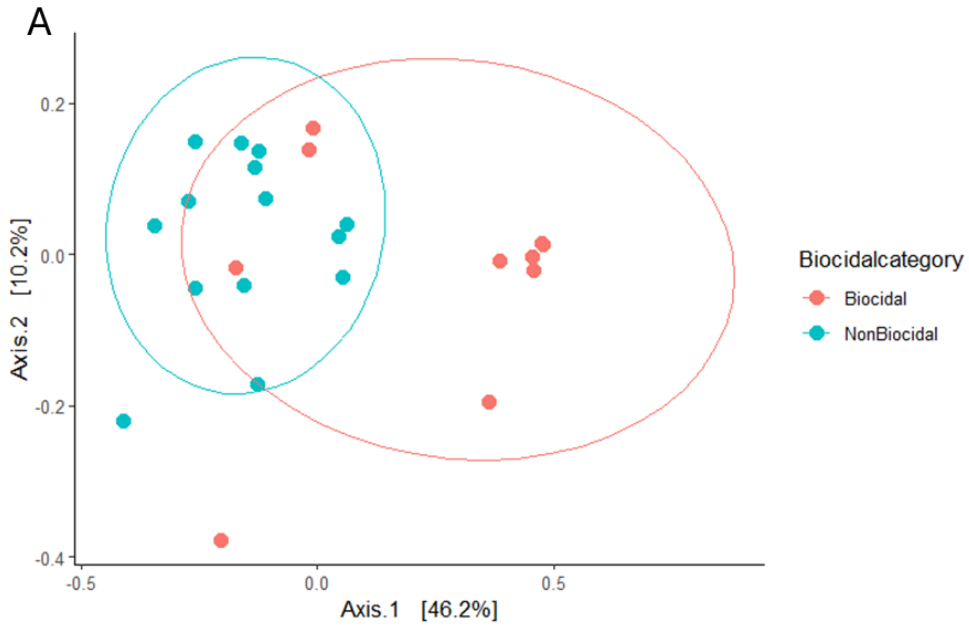


Figure 4.10 C. intestinalis microbiome Bray-Curtis PCoA by biocidal category. (ANOSIM, R=0.4907, p=0.001).

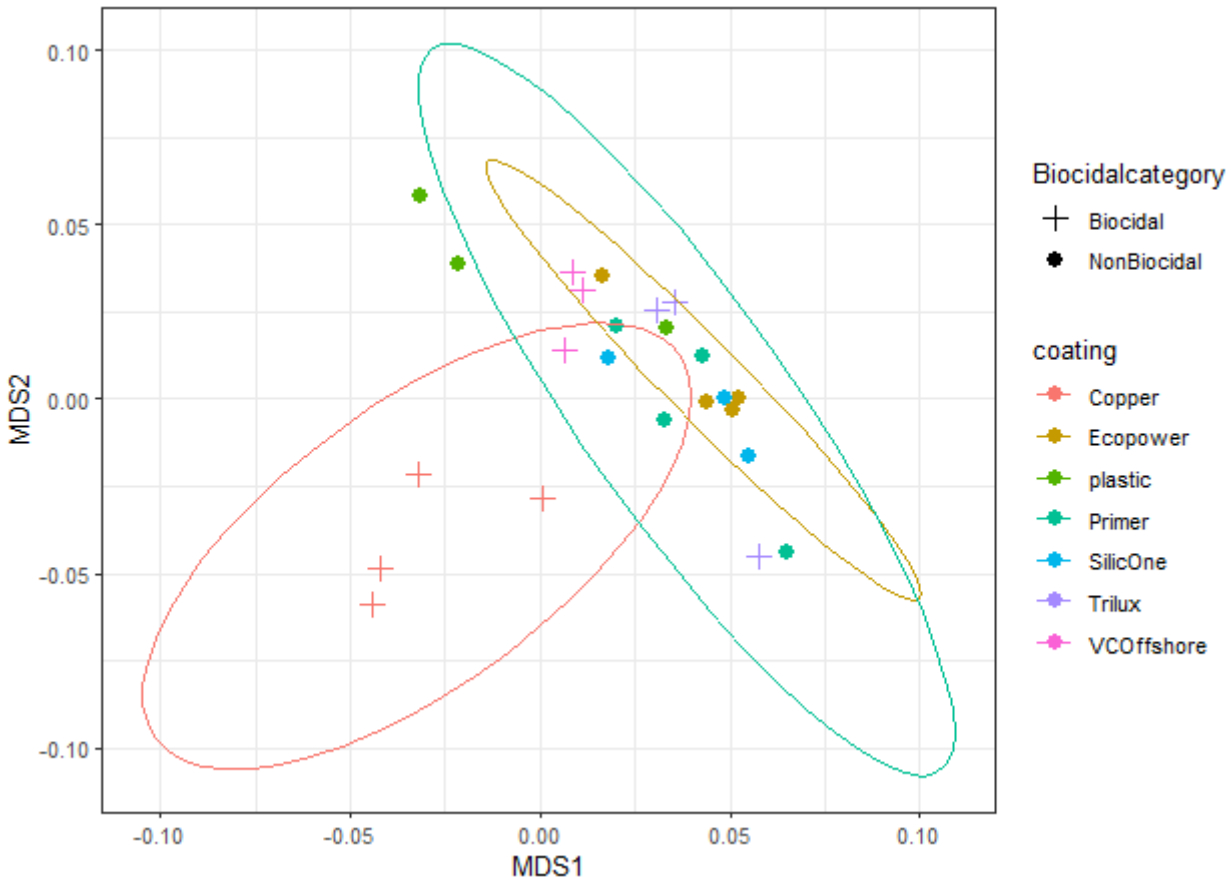


Figure 4.11 C. intestinalis PICRUSt2 Bray-Curtis dissimilarity MDS plots by coating (colour) and biocidal category (shape). (Adonis, by biocidal category: R2=0.28, p=0.004)

4.3.3 *Bugula neritina* Microbiome

The microbial community associated with *B. neritina* was dominated by 15 taxa, contributing more than 2% of the overall community. In general, the composition of the replicates was relatively consistent within treatments. However, for the VC Offshore and Trilux coatings, fewer replicates were available, with the sample from the Trilux coating demonstrating large proportional differences between the two available replicates.

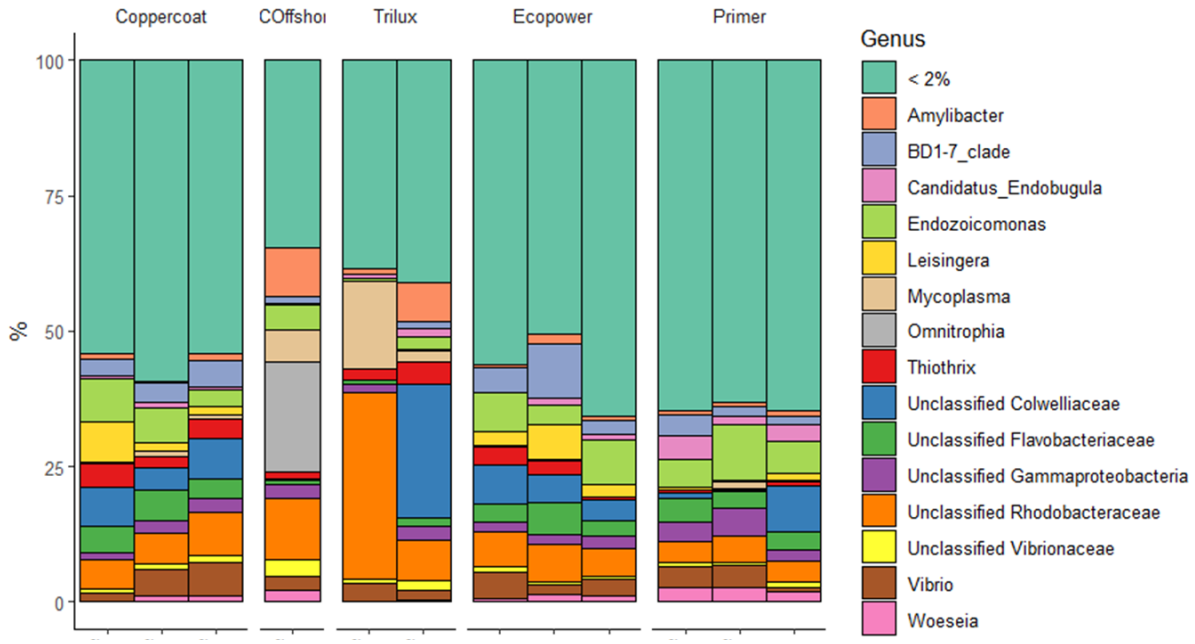


Figure 4.12 Genus level taxonomic profile of B. neritina from different coatings above 2% relative abundance

At the phylum level, only Cyanobacteria and Crenarchaeota were identified as significantly different in the *C. intestinalis* microbiome between biocidal and non-biocidal treatments. For Cyanobacteria, the differences were specifically driven by abundance associated with samples from the biocidal Coppercoat coating (Figure 4.13), whereas the differences in the Crenarchaeota were driven by the non-biocidal Primer coating.

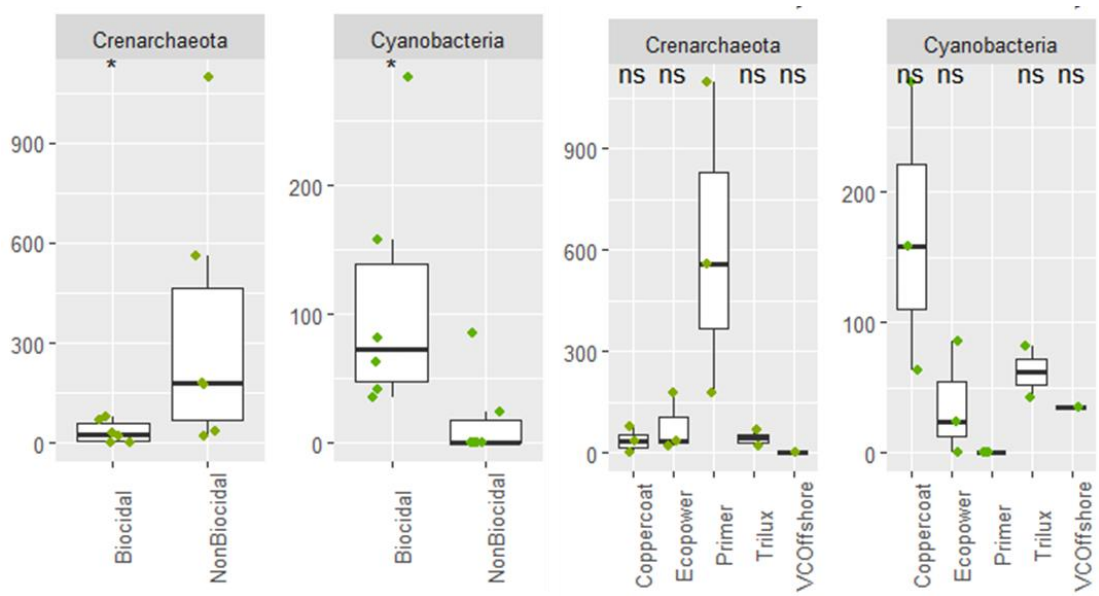


Figure 4.13 Boxplots of significantly different phyla found within B. neritina microbiome samples between biocidal and non-biocidal treatments. Full phylum comparisons by coating in Supplementary Figure 4.2.

DESeq2 differential abundance analysis revealed there were three distinguishable groups of associated taxa that are grouped by biocidal category (Figure 4.14). Group 1 includes all the non-biocidal coatings plus one Coppercoat replicate. Groups 2 and 3 consist of the biocidal coatings, split into the copper-only coating (group 2) and the mixed biocidal coatings (group 3).

The taxa that were differentially abundant in the group 1 non-biocidal coatings were: *Vicingus* sp., *Fabibacter* sp., *Woeseia* sp., *Candidatus_Endobugula* sp., *Reichebbachiella* sp., and JTB23 gamma sp.. When all the non-biocidal coatings were grouped together, it was apparent that the primer coating was driving the differential abundance. Group 2 (Coppercoat) differentially abundant species were: *Crocinitomicaceae* sp., Cellvibronaceae sp., DEV007 sp., and *Crocinitomix* sp.

The differential abundant taxa in mixed biocidal coatings group 3 (VC Offshore and Trilux) were *Winogradskyella* sp., *Mycoplasma* sp., uncultured sp., *Francisella* sp., *Neptuniibacter* sp., *Pontibacterium* sp., *Amylibacter* sp., *Rickettsiella* sp., *Nitrosomonas Nitrosospira*., *Candidatus_Navis* sp.

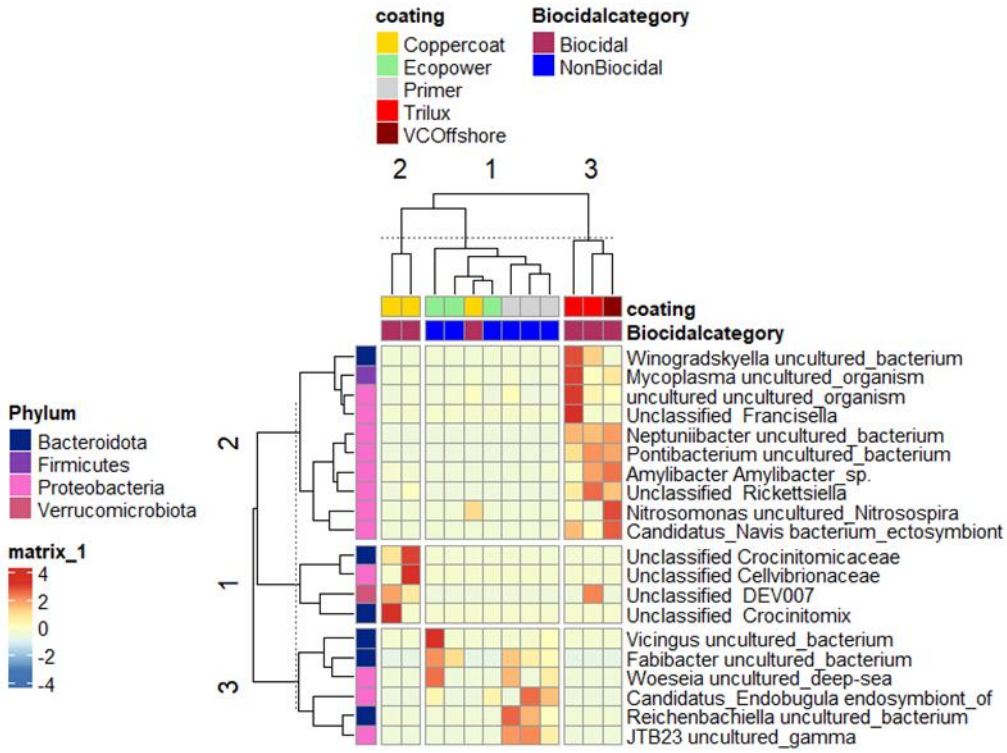


Figure 4.14 DESeq2 differential abundance heatmap of microbial taxa in B. neritina microbiome from different coatings.

Bray-Curtis dissimilarity PCoA demonstrated a clear community shift between the biocidal and non-biocidal treatments. Although there was some overlap, they were statistically different as a group (ANOSIM p=0.004) (Figure 4.15).

Using predicted functionality, based on community compositions, there was no differential shift in the KEGG orthologs between biocidal and non-biocidal coatings (Figure 4.16), indicating similar functional profiles between the two treatments.

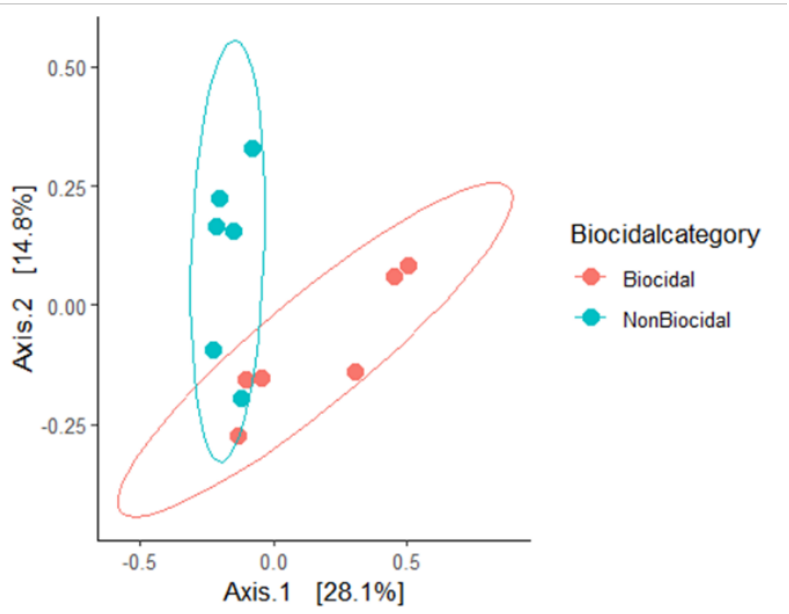


Figure 4.15 B. neritina community Bray-Curtis dissimilarity PCoA by biocidal category. ANOSIM R=0..2769, p=0.004.

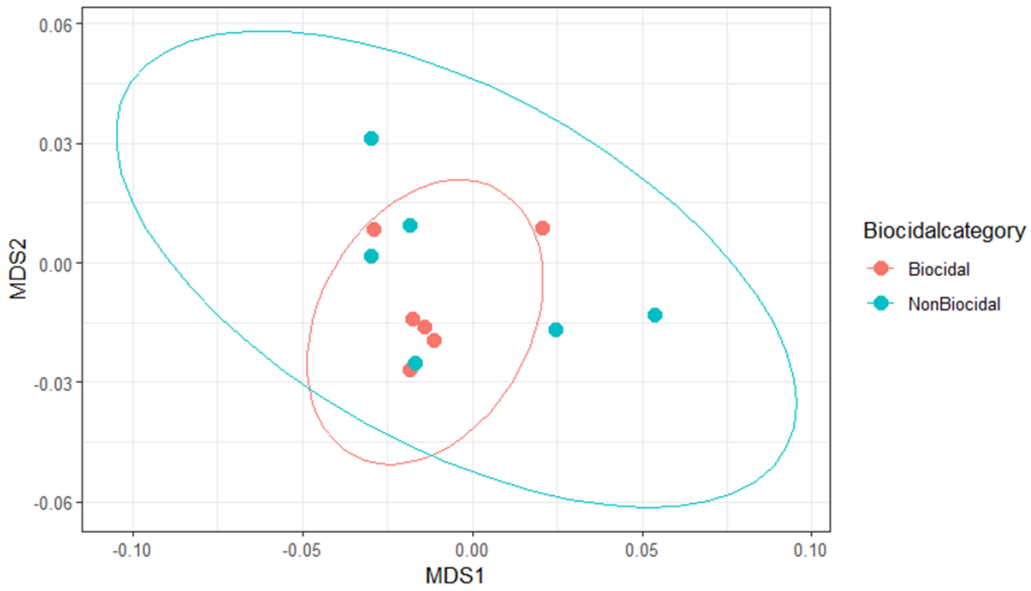


Figure 4.16 B. neritina predicted KEGG profile Bray-Curtis dissimilarity MDS by biocidal category.

4.3.4 Whole Microbiome Culture Resistance Assays

Ciona intestinalis microbiome cultures demonstrated no significant differences in colony counts between different antibiotic treatments (Figure 4.17). *Bugula neritina* microbial communities were, however, susceptible to ampicillin and ciprofloxacin from the biocidal treatments, and to kanamycin susceptibility from the non-biocidal treatments (Figure 4.17).

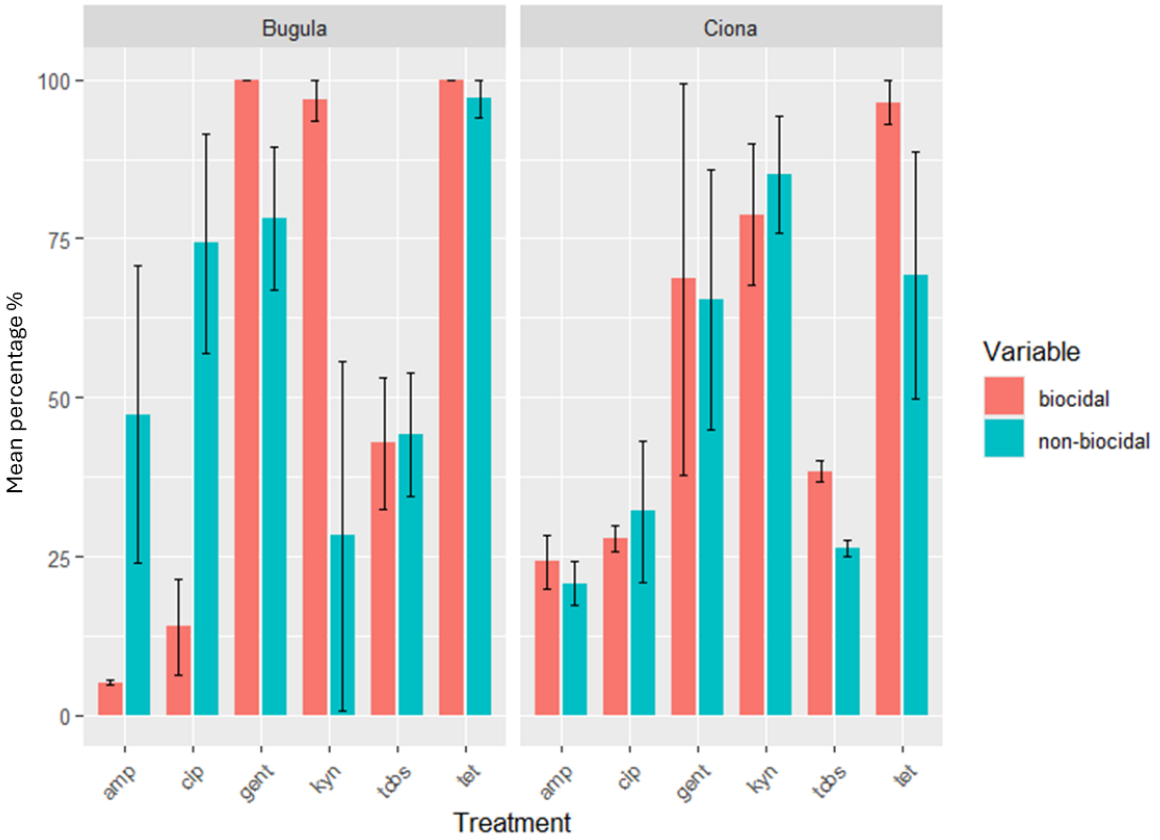


Figure 4.17 Whole microbial community colony counts converted to percentages on antibiotic plates for C. intestinalis and B. neritina microbiomes.

4.4 Discussion

Marine invertebrate microbiomes are diverse and generally demonstrate species specificity in their compositions (dominant taxa and abundances) (Figure 4.5) (Cahill *et al.*, 2016; Galià-Camps *et al.*, 2023; López-Legentil *et al.*, 2023). Although host-associated microbiomes generally demonstrate stability, this study has illustrated that marine invertebrate microbiomes are influenced by the substrates on which they have settled on. It was expected that the communities on biocidal coatings would be less diverse, as has been demonstrated in previous biofilm studies (Briand *et al.*, 2017; Winfield *et al.*, 2018; Catao *et al.*, 2021). However, in this study, it was found that in some invertebrate species, diversity increased for certain biocidal treatments (Figure 4.4). This can be seen in more detail for the *Ciona intestinalis* microbiome community composition, for which a large proportion of the community pooled together under the category “less than 1%”, suggesting that there are a large number of taxa with low abundances. Similar results were found in a study of areas with high anthropogenic impact where alpha diversity was higher than in less impacted areas, which was attributed to the input of organic matter, increasing the microbial load (Jurelevicius *et*

al., 2021). In the context of this study, the higher diversity in biocidal treatments may help with resistance against the biocidal pressures, which allows for similar functional profiles to arise on the biocidal and non-biocidal coatings (Pereira, Pilz-Junior and Corção, 2021). Even with the large variation across species-specific microbiomes, there were some significant predicted functions identified between the biocidal and non-biocidal treatments (Figure 4.6). The former showed an abundance of KEGGs related to manganese/zinc/iron transport systems, while non-biocidal treatments showed an abundance of KEGGs related to V/A-type H+/Na+ transporting ATPase subunits.

4.4.1 The *Ciona intestinalis* Adult Microbiome

Microbiome analysis of *C. intestinalis* demonstrates species-specific microbiome conservation, aligning with other studies (Blasiak *et al.*, 2014; Dishaw *et al.*, 2014; Cahill *et al.*, 2016; Utermann, Blümel, *et al.*, 2020) and coating/environment-specific adaption (Utermann, Blümel, *et al.*, 2020). Most studies have used tissue-specific analysis, whereas this study took an organism 'global' approach. However, commonalities and trends are still seen in the community compositions. The most dominant taxa were Proteobacteria, followed by Cyanobacteria, which were found in most gut samples of other studies or in the tunic if from polluted environments (Dishaw *et al.*, 2014; Utermann, Blümel, *et al.*, 2020). Actinobacteria was also a dominant taxon, which was also found in Utermann *et al.* (2020) who also used 16S sequencing to microbiome community profile gut and tunic samples of *C. intestinalis*. This study found large relative abundances of Planctomycete, which is common in small amounts (gut and tunic) in other studies of *C. intestinalis* microbiomes, whereas it was highly relatively abundant in the mixed-biocidal samples in this study suggesting coating/environment-specific changes.

There were also microbial community differences between the biocidal and non-biocidal coatings (Figure 4.10, Figure 4.9 and Figure 4.8). The majority of these differences were driven by two of the three biocidal coatings, specifically the mixed biocide coatings, Trilux and VC Offshore. The Coppercoat coating is exclusively based on copper filings, and demonstrates more similarities with the non-biocidal coatings with regard to its dominant taxa (Figure 4.7), suggesting that copper alone doesn't have the same disruptive effect as the other coatings.

There were seven phyla that were statistically more abundant in the biocidal treatments (Acidobacteriota. Actinobacteriota, Euryarchaeota, Verrucomicorbiota, Dependentiae, Gemmatiomanodota and Planctomycetota), and two more abundant phyla on the non-biocidal treatment (Bdellovibrionota and Proteobacteria) (Figure 4.8). Acidobacteriota, which was found more on the biocidal treatments, have a rich natural product potential in sponges (Leopold-Messer *et al.*, 2023). Some Actinomycetota species are also of medical or economic significance; many of which can cause disease in humans, while also being a notable source of antibiotics (de Lima Procópio *et al.*, 2012). All cultured members of Euryarchaeota, a phylum of the Archaea, are thermophilic or hyperthermophilic organisms and include methanogens, halophiles, and sulfur-reducing thermophiles (Gaasterland, 1999). Dependentiae is found in various environments worldwide, and previous genomic evidence suggests it has ancestral and unusual adaptations to a host-dependent lifestyle. Overall, however, there is little knowledge about this phylum. Because the majority of its group is yet-to-be cultivated, it is often classed as 'Microbial Dark Matter' (MDM) (Weisse *et al.*, 2023). Gemmatiomanodota and Planctomycetota both play crucial roles in carbon and nitrogen cycling, which could be beneficial for host-microbe interactions and environmental health (Delmont *et al.*, 2018; Abreu *et al.*, 2022; Li *et al.*, 2023). Members of the phylum Planctomycetota are ubiquitously present in the environment including in hotspots for antimicrobial resistance selection (Godinho *et al.*, 2024).

The most notable observation from the *C. intestinalis* microbiomes from the biocidal coatings at the genus level was the major reduction in particular taxa, specifically *Endozoicomonas* spp. from the VC Offshore and Trilux coatings (Figure 4.7). *Endozoicomonas* spp. are prevalent and abundant bacterial associates of various marine animals (Neave *et al.*, 2016). It has been proposed that *Endozoicomonas* spp. plays an important role in holobiont nutrient cycling and, therefore contribute to host health, acclimatisation, and adaptation (Neave *et al.*, 2016; Pogoreutz *et al.*, 2022; Pogoreutz and Ziegler, 2024). If *Endozoicomonas* sp. provides similar functions in *C. intestinalis*, then its absence may indicate reduced health or ability to deal with extra stresses. How *Endozoicomonas* spp. functions in other host-microbiomes are still unclear, but it has been documented in other cnidarians (Tinta *et al.*, 2019), sponges (Nishijima *et al.*, 2013), molluscs (Beinart *et al.*, 2014), worms (Forget and Kim Juniper, 2013), fish (Mendoza *et al.*, 2013; Katharios *et al.*, 2015) and *Ciona* sp. (Dishaw *et al.*,

2014; Cahill *et al.*, 2016). It has been suggested that *Endozoicomonas* spp behaves as a facultative symbiont in *Ciona* sp. and other ascidian species. The ascidian-specific subclade of *Endozoicomonas* has also been loosely suggested to be acquired through horizontal transmission from the surrounding environment, although no other study has definitely confirmed this (Schreiber *et al.*, 2016). In some species of corals, the higher association of *Endozoicomonas* has actually been linked with decreased ability for environmental adaptation (Neave *et al.*, 2017; Pogoreutz and Ziegler, 2024). In mollsucs and fish it is an emerging and prolific pathogen (Katharios *et al.*, 2015). Therefore, further investigation of the role of *Endozoicomonas* in fouling invertebrates would be worthwhile, particularly in the context of the present study, to determine if any pathogenic species are being harboured and transported within these communities. If this is the case, and *Endozoicomonas* sp. is being eradicated from invertebrate microbiomes on biocidal coatings, it would highlight the benefit of anti-fouling coatings in relation to bio-security and reducing the spread of particular pathogens. This would need extensive further testing.

Interestingly, *Vibrio spp.* was also absent from the two mixed biocidal coatings (VC Offshore and Trilux) (Figure 4.7). *Vibrio* not only contains many pathogens of interest to human and environmental health (Baker-Austin *et al.*, 2018), but has also been demonstrated to provide crucial host-symbiont functions (Rubiolo, Botana and Martínez, 2019; Diwan, Harke and Panche, 2023; Ma *et al.*, 2023), which those in the biocidal-treated *Ciona* microbiomes were lacking. It is likely that these microbial species provide beneficial functions to their host, and that those *C. intestinalis* on the biocidal coatings may therefore be of reduced health. Predicted functional profiles on the initial inspection are compositionally statistically different between biocidal and non-biocidal treatments (Figure 4.11); however, those differences are actually influenced by the Coppercoat treatment only and not the mixed biocidal coatings (Trilux and VC Offshore), suggesting that even though the community composition is quite different, they still display similar predicted functions (Figure 4.11). This is also supported by the antibiotic susceptibility tests on whole microbiome communities of *C. intestinalis*, where antibiotic susceptibility/resistance to the various antibiotics tested were relatively unchanged between the biocidal and non-biocidal treatments (Figure 4.17).

Host health was not assessed in this study; however further study on these bacterial associations e.g. *Vibrio* and *Endozoicomonas*, and host health would also create a deeper picture of the complex dynamics of anti-fouling technology and its effects beyond standard toxicity. Further assessment using metabolomics and transcriptomics would also provide a deeper insight into any changes in the functionality of the host microbiomes.

4.4.2 The *Bugula neritina* Adult Microbiome

Microbiome analysis of *B. neritina* has highlighted similar dominant phyla as reported in a previous study, namely, Gammaproteobacteria, Alphaproteobacteria and Bacteroides groups (Hai Li *et al.*, 2019). *B. neritina* community compositions of dominant taxa were generally consistent across the non-biocidal coatings and the biocidal, copper-only, Coppercoat coating. The samples from the remaining biocidal treatments displayed a lot of variation in community composition (Figure 4.12). In general, the dominant taxa consisted of *Vibrio* sp., *Rhodobacteraceae* sp., *Flavobacteriacaece* sp., *Colwelliaceace* sp., *Thiothrix* sp., *Leisingera* sp., *Endozoicomonas* sp. and BD1-7 clade sp. The mixed biocidal coatings, VC Offshore and Trilux, were highly variable but did demonstrate higher rates of *Amylibacter*, *Mycoplasma* and *Rhodobacteraceae*. Beta diversity Bray-Curtis dissimilarity analysis confirmed the differences in community composition between biocidal and non-biocidal coatings, specifically with the VC Offshore and Trilux coatings driving the differences (Figure 4.15). However, based on the diversity of the predicted functional profiles, created using the PICRUSt2 pipeline, there were no significant differences in their functional profiles (Figure 4.16). The trend of pollutant-affected microbiomes displaying different compositions, but similar functionality has been seen in several studies (Pereira, Pilz-Junior and Corção, 2021; Peña-Montenegro *et al.*, 2023). As mentioned in the previous section with *C. intestinalis*, this could be an adaptation to the stressors (biocides), creating a community that is functionally similar but with better resistance to the biocides.

However, whole microbiomes of *B. neritina* from biocidal coatings, were more susceptible to the antibiotics ampicillin (beta-lactam) and ciprofloxacin (quinolone). Alternatively, those from the non-biocidal coatings were sensitive to kanamycin (aminoglycoside). This illustrates that the susceptibility of microbiomes to antibiotics

are influenced by biocidal anti-fouling coatings, and that their potential resistance profiles are dynamic.

The mixed biocidal coatings had the most differentially abundant taxa, of which *Mycoplasma* was one. *Mycoplasma*, as a genus, contains 200 species including five major pathogens that have been profiled for their antibiotic resistance. 16S rRNA sequencing was unable to identify to the species level in this study; therefore, the supplementary shotgun sequencing that is to follow this thesis could reveal which species they are, and if they are the pathogenic species of concern.

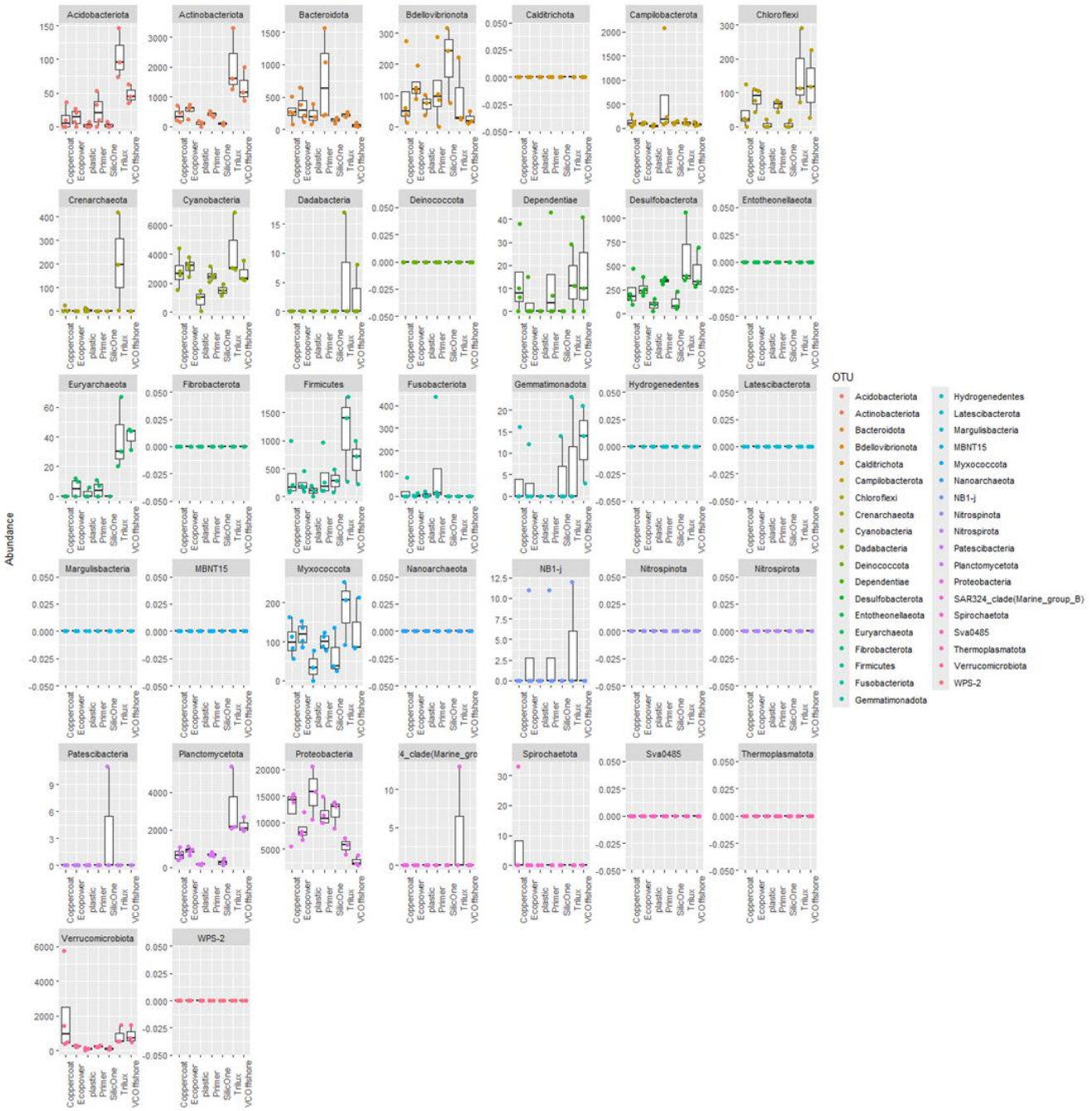
One noticeable difference found in the *B. neritina* microbiomes on the anti-fouling coatings, compared to the Primer coating was the reduction in the relative abundance of *Endobugula*. For the anti-fouling coatings (biocidal and the non-biocidal Ecopower), the relative abundances were <1% of total community composition, whereas on the primer control coating, it ranged from >1-4% of community composition (Figure 4.12, Supplementary Figure 4.3). *Endobugula* is a crucial endosymbiont of *B. neritina* due to its ability to produce bryostatins, which acts as a chemical defence against predatory activity or pathogens (R M Woollacott, 1981; Lopanik, Lindquist and Targett, 2004; Sharp, Davidson and Haygood, 2007). The reduction of *Endobugula* could be indicative of a reduced ability to produce these essential beneficial compounds, and thus reduced fitness. Further investigation could quantify the production of bryostatins from *B. neritina* on different coatings and assess *B. neritina's* fitness, and whether they become more susceptible to disease and/or predation. This could then have potential impacts on larval health and survival if *Endobugula* is not transferred at its optimal rate (Chapter 5).

4.4.3 Conclusion

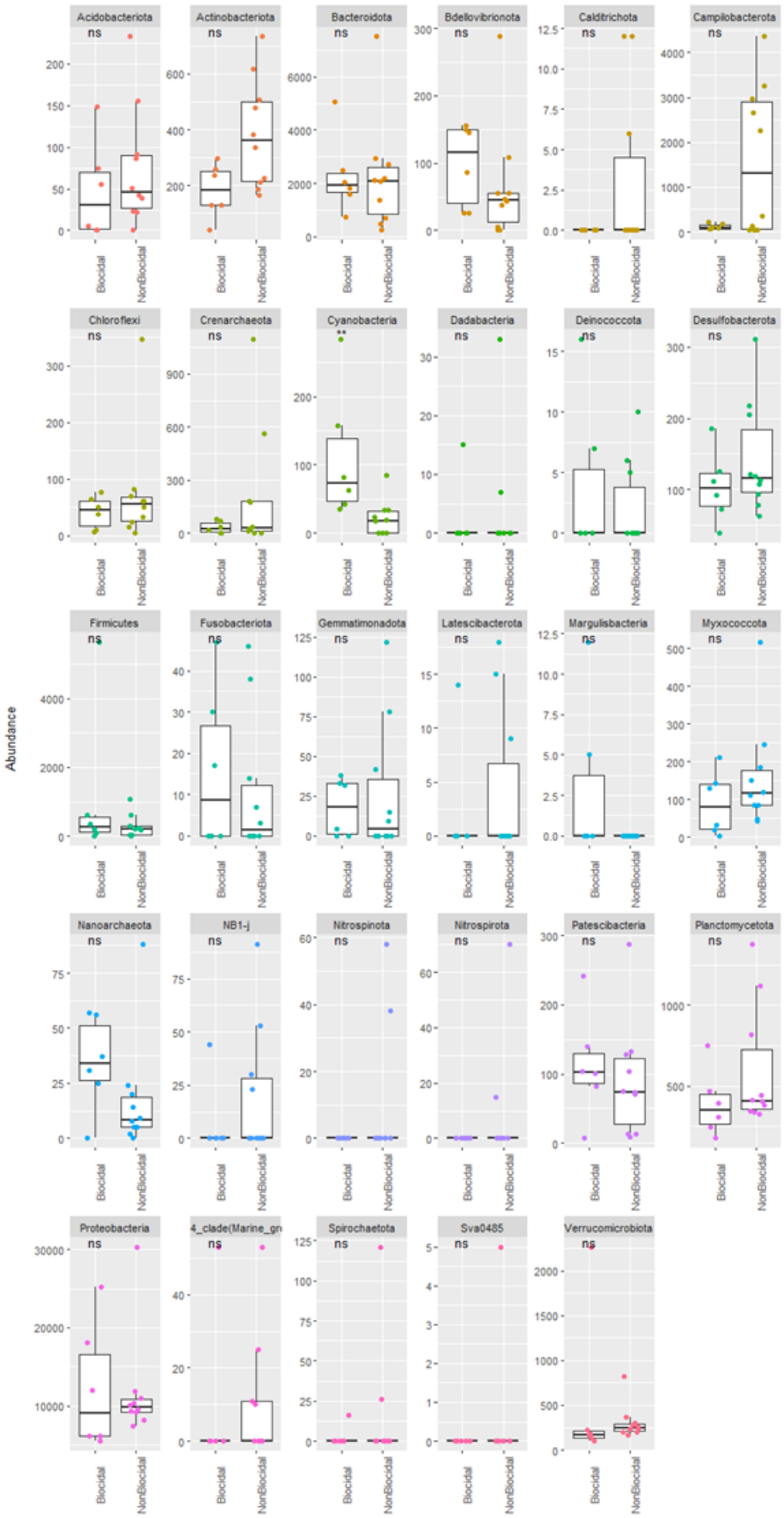
It is evident that anti-fouling coatings can cause compositional changes within the microbiomes of marine fouling invertebrates. Biocidal treatments in general exhibited a reduction in potentially beneficial microbial taxa associated with the hosts. However, even with statistically different community compositions, overall diversity and predicted functionality of those microbiomes appear to remain similar in *C. intestinalis* and *B. neritina*. However, in *B. neritina*, different antibiotic susceptibilities were identified between the biocidal and non-biocidal coatings, with those on biocidal coatings exhibiting beta-lactam (ampicillin) and quinolone (ciprofloxacin) sensitivity, while non-

biocidal microbiomes exhibited aminoglycoside susceptibility. This suggested that biocidal treatment may reduce resistance against the antibiotics tested. This poses interesting questions regarding the mechanisms that are involved, what microbial taxa may be driving these differences, and the implications of varying resistance profiles. Future studies with microbial isolates from these microbiomes and extensive antibiotic resistance assays would reveal more information on the resistance profiles of culturable taxa associated with anti-fouling biocides.

4.5 Supplementary



Supplementary Figure 4.1 Phyla level comparisons of relative abundance in C. intestinalis microbiomes across each coating tested.



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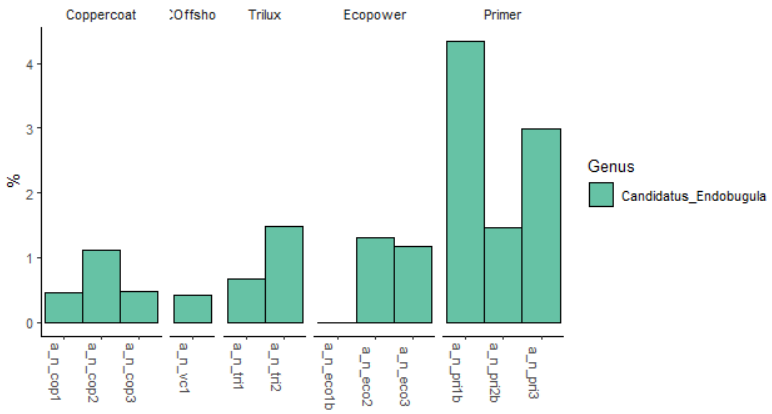
2454 *Supplementary Figure 4.2 Phyla level comparisons of relative abundance in B. neritina*
2455 *microbiomes between biocidal and non-biocidal treatments.*



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2457 *Supplementary Figure 4.3 Relative abundance of Candidatus_endobugula only from*
2458 *B. neritina microbiomes*

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Chapter 5: Microbiome Transfer from Fouling Invertebrate Adults to Larvae

5.1 Introduction

Microbial symbioses are widespread phenomena which play functionally important roles in the life history and development of many invertebrates (R. M. Woollacott, 1981; Giere and Langheld, 1987; Klussmann-Kolb and Brodie, 1999; Sipe, Wilbur and Cary, 2000; Lopanik, Lindquist and Targett, 2004; Salerno *et al.*, 2005; Maldonado, 2009; Apprill *et al.*, 2012; Sharp, Distel and Paul, 2012; Guri *et al.*, 2012; Björk *et al.*, 2019; Vijayan *et al.*, 2019; Carrier and Reitzel, 2019, 2020; Freire *et al.*, 2019). Together, the host and its associated microbial community form its own synergistic ecological unit, termed the holobiont (Dheilly, 2014), often providing mutualistic and environmental function (Trevathan-Tackett *et al.*, 2019).

The health of offspring is often influenced by the parental microbiome (Nyholm, 2020; Paniagua Voirol *et al.*, 2020; Bunker *et al.*, 2021; Benítez-Páez, 2023; Schneider *et al.*, 2024) and thus host-generational changes to the microbiome may have further implications for the newer generation's ability to resist stressors such as disease, stress, and climate change (Marangon *et al.*, 2023), as well as the transfer of pathogens (Frank *et al.*, 2008; Wilson *et al.*, 2021). Maternal transmission of microbiota has been well documented in mammalian species (Mirpuri, 2021) and, more recently, oviparous species such as reptiles and birds where the mechanisms of maternal microbiome transfer are diverse between species (Murphy *et al.*, 2023). In general though, the early inoculation of microbes is critical for healthy development, and to 'seed' the adult microbiome, with long-term health consequences if disrupted (Knutie *et al.*, 2017; Shao *et al.*, 2019; Warne, Kirschman and Zeglin, 2019; Kim *et al.*, 2020; Yoon *et al.*, 2022).

Invertebrates encompasses highly diverse taxonomic groups with a broad range of life history traits and reproductive strategies. Microbiome acquisition by invertebrate larvae can involve both vertical (maternal) and horizontal transmission of symbionts (transmission between members of the same species or from the environment) (Bright and Bulgheresi, 2010). Symbiont transmission varies across species, but has been well documented in some invertebrates such as insects (Coon, Hegde and Hughes, 2022), nematodes (Dirksen *et al.*, 2016) and the bobtail squid *Euprymna scolopes*

(Nyholm and McFall-Ngai, 2004). Marine invertebrates have diverse life cycles, and the point of microbiome acquisition is a relatively new area of study with many knowledge gaps. Popular species for research include bio-active rich sponges (Maldonado, 2009; Webster, N.S.; Taylor, M.W.; Behnam, F.; Lücker, S.; Rattei, T.; Whalan, S.; Horn, M.; Wagner, 2010; Björk *et al.*, 2019; de Oliveira *et al.*, 2020), commercially important oysters (Lokmer and Wegner, 2015; Unzueta-Martínez *et al.*, 2022; Scanes *et al.*, 2023), and coral species (Lesser, 1997; Rohwer, F.; Seguritan, V.; Azam, F.; Knowlton, 2002; Thurber *et al.*, 2009; van de Water *et al.*, 2018; Wilkins *et al.*, 2019).

In terrestrial insects, bacterial symbionts are predominantly vertically (maternally) transmitted (Ferrari and Vavre, 2011). In sponges, microbiome uptake has been shown to happen both vertically and horizontally (Björk *et al.*, 2019; Sacristán-Soriano *et al.*, 2019; Díez-Vives *et al.*, 2022). Similarly, in the Sydney Rock Oyster, microbiomes are acquired through both vertical and horizontal transmission, with different larval stages influenced by seawater microbiomes and specific oyster-associated microbes, often transmitted through the egg (Unzueta-Martínez *et al.*, 2022; Scanes *et al.*, 2023). In invertebrates like the honey bee, microbiome transfer after emergence overrides variations in the larval microbiome, indicating decoupling of larval and adult microbiome stages (Kowallik and Mikheyev, 2021). Additionally, in arthropod vectors like ticks, the microbial community may include pathogens transmissible to other hosts (Varela-Stokes *et al.*, 2018; Adegoke *et al.*, 2022). These studies highlight the diverse mechanisms of microbiome transfer in invertebrates, ranging from the maternal transmission to the environmental acquisition, and that microbiomes are dynamic and are species and life-stage-specific.

Microbiome transfer may be influenced by external factors such as ocean acidification (Zhou *et al.*, 2021; Scanes *et al.*, 2023) and climate change (Luter *et al.*, 2020; Zhou *et al.*, 2021). The diet of the host can also influence microbiomes of marine invertebrates, enabling enhanced nutrient extraction from specific food sources (Huang *et al.*, 2020; Feng *et al.*, 2023). Sub-optimal diets have been demonstrated to cause microbial shifts and reduced fitness in oyster larvae (Vignier *et al.*, 2021), black solider flies (Auger *et al.*, 2023) and crown of thorns seastars (Carrier *et al.*, 2018) among others (Triggs and Knell, 2012; Wilkes Walburn *et al.*, 2019; Kroetsch *et al.*, 2020; Mirpuri, 2021). The functionality of host-associated microbiomes have been

evidenced to play key ecological roles such as nutrient cycling, disease prevention and health of the host.

Understanding the microbial composition of invertebrate holobionts and whether they are transferred trans-generationally helps further the understanding of these relationships, as well as providing insight for potential biosecurity risk, especially in circumstances of species transfer of e.g. marine biofouling invertebrates. Fouling organisms may pose extra risk due to their ability to travel on 'vessels of opportunity' and survive exposure to anti-fouling biocides. Even though adults will remain attached to their original substrate, reproduction through asexual or sexual reproduction may allow the passing on of their microbiome and any potential undesirable genes into the surrounding environment.

This study aimed to assess the trans-generational microbiome transfer of four marine biofouling species (*Ciona intestinalis, Bugula neritina, Botrylloides violaceus* and *Amphibalanus improvisus*) using 16S rRNA sequencing by comparing community compositions and 'tracking' the microbial communities from parent/adult to the dispersal stage (offspring/larvae).

5.2 Methods

5.2.1 Sample Collection

From August to September 2023, specimens of *Ciona intestinalis*, *Bugula neritina* and *Botrylloides violaceus* were collected from the underside of pontoons from Queen Anne's Battery Marina and Millbay Marina in Plymouth, UK. Specimens were transported in natural seawater and stored in a temperature-controlled room at 18°C, and kept in the dark overnight. Barnacle specimens were provided by the Newcastle biofouling group; laboratory-reared *Amphibalanus improvisus* were used.

5.2.1.1 Ciona intestinalis

Specimens of *C. intestinalis* from Millbay Marina that demonstrated pink egg masses were washed in filtered seawater and left in autoclaved seawater overnight in the dark at 18°C. The next day, individuals were dissected using sterile scissors and forceps to obtain gametes as described in Yunnie (2009) and (Leigh, Liberti and Dishaw, 2016). Gametes were collected using sterile Pasteur pipettes and placed in individual watch glasses in filtered seawater for 10 minutes to mature. Gametes were then cross-

fertilized with the opposite gametes from another specimen and left to fertilise for up to 1 hour. Fertilised eggs were washed to prevent poly-spermy, and then transferred to larger glass containers with more filtered seawater. These were then continuously mixed overnight using a bespoke mixer (Figure 5.1) at 18°C to develop and hatch. Upon successful hatching, which happens 12-24 hours later, larvae were pipetted out and placed in 1.5ml microcentrifuge tubes where they were washed with three rinses of filtered seawater. The adult, unfertilised gametes and successfully hatched larvae were collected and frozen prior to DNA extraction.

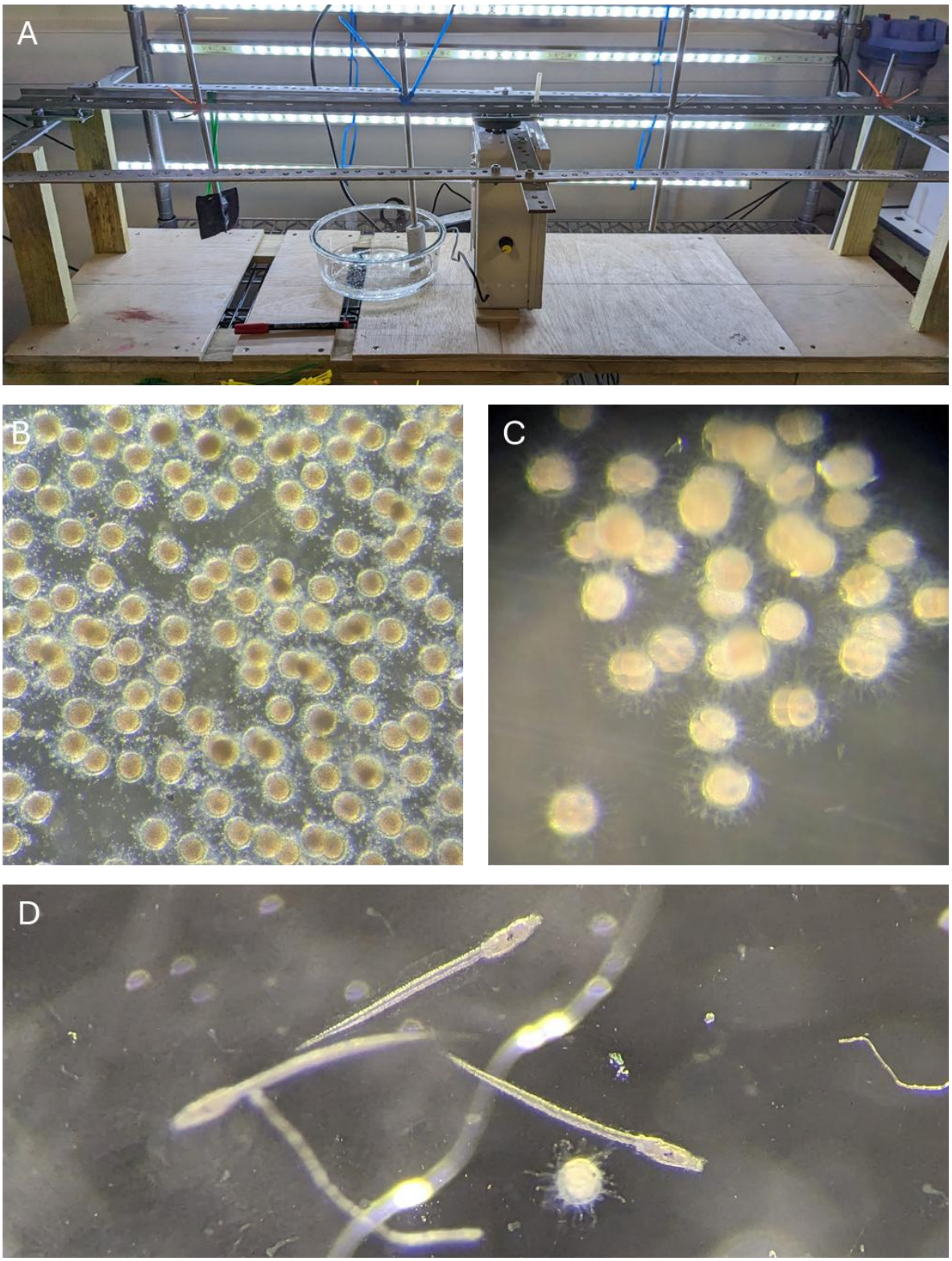


Figure 5.1 Ciona intestinalis larvae setup. (A) the bespoke mixing contraption with autoclavable silicone paddles to keep steady water movement to prevent clumping. (B) unfertilised C. intestinalis eggs (C) fertilised C. intestinalis eggs (D) hatched C. intestinalis larvae

5.2.1.2 *Bugula neritina* and *Botrylloides violaceus*

Both *B. neritina* and *B. violaceus* were induced similarly for spawning. Specimens were both kept overnight in the dark at 18°C. Upon light exposure the following day of collection, larvae spawned within 1-2 hours. Larvae were collected using sterile pasteur pipettes and transferred to sterile 1.5ml microcentrifuge tubes. Post-spawning, portions of the adult from each replicate were dissected using sterile scalpels and placed in 1.5ml microcentrifuge tubes and frozen in preparation for DNA extraction.

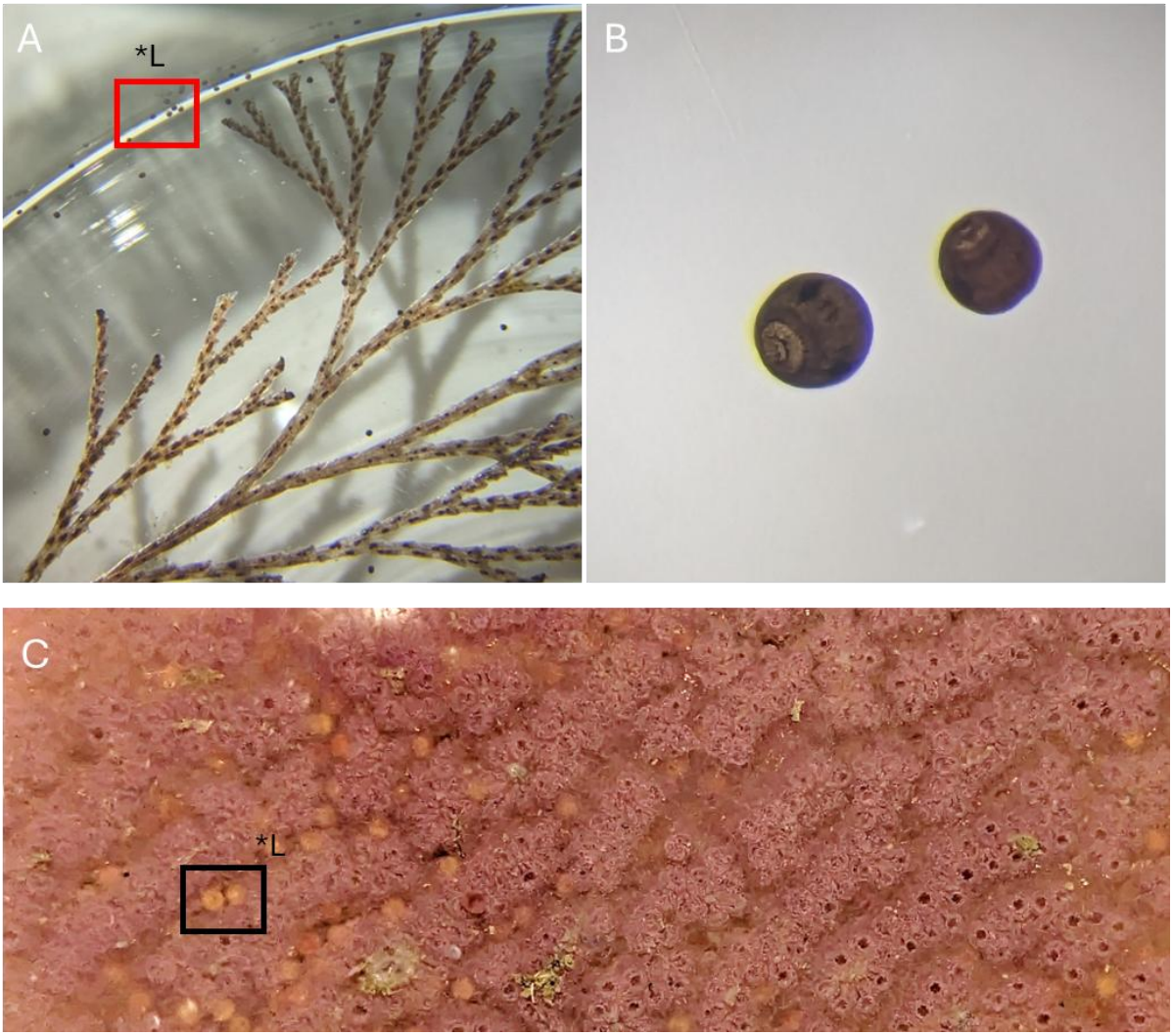


*Figure 5.2 (A) B. neritina adult. The red box highlights the released larvae *L (B) Close-up visual of B. neritina larvae (C) Close up of a B. violaceus adult colony and the pre-released larvae in-situ ready to spawn in the highlighted black box *L*

5.2.1.3 *Amphibalanus improvisus*

A. improvisus were lab-reared, cultured in artificial seawater at 18°C. Their containers were emptied overnight and refilled the next day with autoclaved artificial seawater to

induce spawning. *A. improvisus* were kept in the dark with a small light source to one side of the container to attract released nauplii. Nauplii were collected every 20 minutes for 2 hours using a sterile pasteur pipette and placed in sterile 1.5ml microcentrifuge tubes for each replicate. Samples were then frozen at -20°C in preparation for subsequent DNA extractions. Two to three adults from the respective buckets were removed, frozen and stored for DNA extraction as well as a 500µl seawater sample from each tank.

5.2.2 DNA Extractions

For 16S rRNA analysis, DNA was extracted using the Qiagen Powerlyzer powersoil kit as per manufacturer's instructions. Seawater samples were filtered onto sterile 0.22im filters, and used for DNA extraction. For shotgun sequencing analysis, the Zymo host zero kit was used as per manufacturer instructions. Extracted DNA was sent to NUOMICS (Northumbria University, UK) for quality checks and sequencing.

5.2.3 Sequencing

DNA Preparation and sequencing were conducted through NUOMICs (Northumbria University). PCR was performed using the EMP (earth microbiome project) primer set 515F–806R which targets the V4 region of the 16S SSU rRNA (FWD:GTGYCAGCMGCCGCGGTAA (Parada, Needham and Fuhrman, 2016); REV:GGACTACNVGGGTWTCTAAT (Apprill *et al.*, 2015). Using the pre-barcoded primers (Integrated DNA Technology), a PCR was run under the following conditions: 94°C for 3 minutes, followed by 35 cycles of: 94°C for 45s, 50°C for 60s, 72°C for 90s, then a final extension period of 72°C for 10 minutes. PCR was carried out using 0.8x KAPA 2G Robust Taq polymerase (Roche), 0.2µM of each primer and 1µl of DNA. The PCR products were measured using PicoGreen (Invitrogen) on a Spark plate reader (TECAN), then normalised to a concentration of 50nmol/µl. The library was made by pooling 5µl from each sample together prior to clean up using AMPure XP (Beckman Coulter), followed by two washes using 80% ethanol. The library was resuspended in molecular grade water. Library concentration was remeasured using the high sensitivity DNA kit (Invitrogen) using the Qubit 4.0 (Invitrogen) and the product size was checked using the high sensitivity DNA kit (Agilent) on the Agilent Bioanalyzer 2100. The library was dissociated into single strand DNA using an equal volume of 0.2N sodium hydroxide which was stopped after 5 minutes using 400nM Tris pH 8.0 buffer. The library was then diluted to 20pMol with Illumina hybridisation buffer before

being spiked with 20% v/v PhiX (phage lamda DNA) as in internal quality control. The final library was loaded into the Illumina MiSeq v2 500 cycle reagent cartridge.

5.2.4 Bioinformatic Analyses

Quality-filtered, demultiplexed fastq files were processed using Qiime2-2023.2 (Bolyen *et al.*, 2019). Sequences were quality trimmed and denoised using DADA2 (Callahan *et al.*, 2016) based on the 25th percentile at a minimum of 30 x quality score (forward 243, reverse 14 to 187) producing unique sequences (2429 features over 43 samples, 669,285 total reads). Chimeras were identified and removed using UCHIME (Edgar *et al.*, 2011), along with very low abundant features (less than 10). Sequences were aligned to the v4 region of the 16S rRNA gene sequences from the SILVA v138 database (Quast *et al.*, 2013). Non-desirable sequences (those classified as eukaryote, mitochondria, archaea, chloroplast or unknown according to silva taxonomies) were removed. A phylogenetic tree was built using fast-tree. Feature tables, taxonomies, rooted tree and metadata were exported for downstream analysis in RStudio (R Core Team, 2023). Prior to further analysis, all data was 'SCRuB'ed (Austin *et al.*, 2023) to remove any potential introduced contamination based on the associated negative controls. The resulting feature table is what was used for data analysis. Data were then separated by species and rarefied by the lowest sample read (*B. violaceus* = 11413, Barnacle = 5724, *B. Neritina* = 6222, *C. intestinalis* = 3738). Diversity metrics and core microbiome taxa identification were conducted using Phyloseq (McMurdie and Holmes, 2013) and Vegan (Oksanen *et al.*, 2024) packages. Differential analysis was conducted using DESEQ2 (Love, Huber and Anders, 2014). Microbiome source tracking was conducted using Sourcetracker 1.0 (Knights *et al.*, 2011)

5.3 Results

5.3.1 Colonial Tunicate: *Botrylloides violaceus*

Based on the number of unique ASVs (Figure 5.3A), there were more shared ASVs between the adults and larvae of *B. violaceus* than larvae and the seawater. Unique ASVs made up similar relative abundance totals between each stage, contributing to less than 1% of the total community (Figure 5.3D). Larvae shared 31.9% of their ASVs with their parents, and 7.8% with the surrounding water. From a diversity perspective, larvae were generally more diverse (Shannon, Simpson, InvSimpson) (Figure 5.3B). Beta diversity demonstrated clear differences between the communities, suggesting distinct compositions (Figure 5.3C).

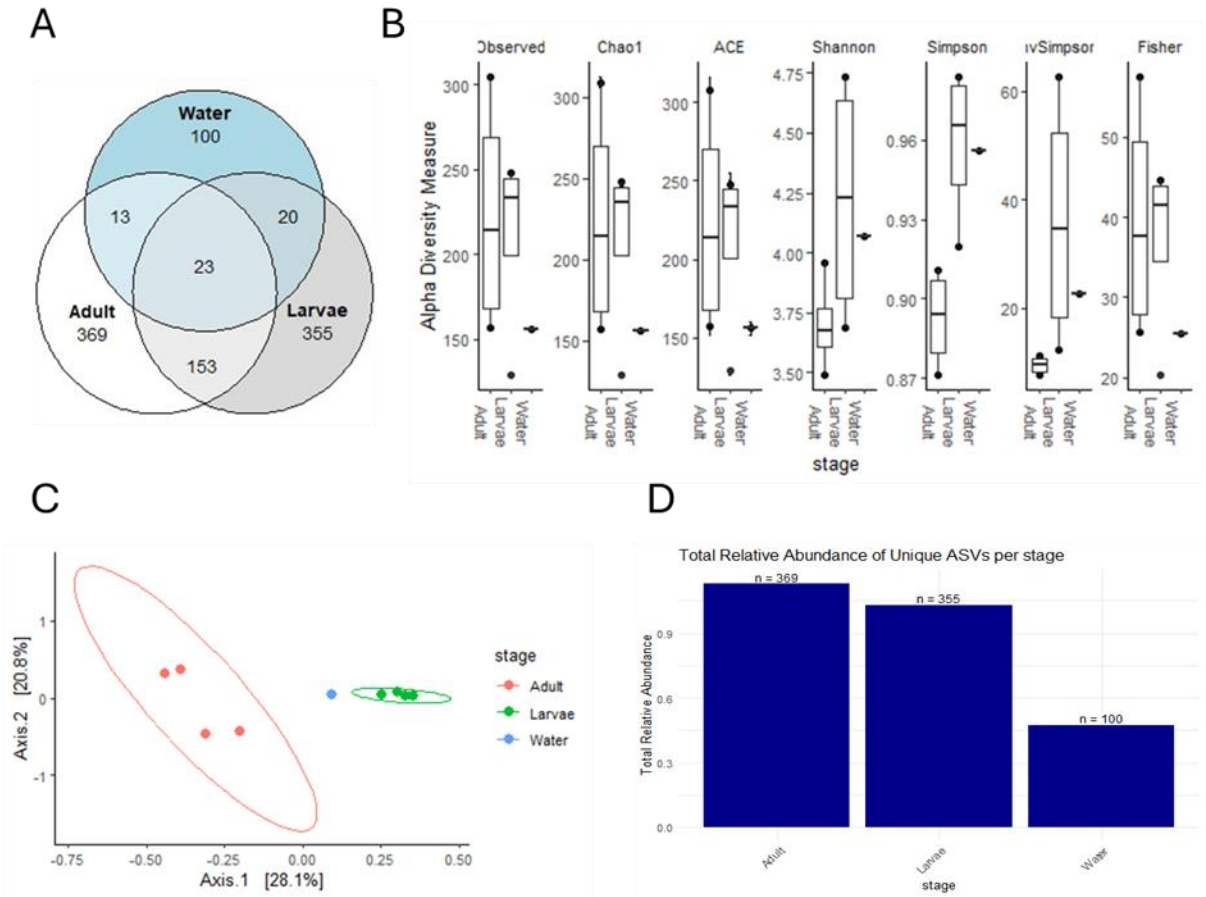


Figure 5.3 (A) Venn diagram of unique ASVs from B. violaceus adults, larvae and the surrounding seawater. (B) Alpha diversity and richness from adults, larvae and the surrounding seawater (C) Jaccard beta diversity PCoA plot. (D) total relative abundance of unique ASVs per stage plot.

Relative abundance community composition demonstrated compositional differences in the community makeup, with different taxa being more dominant between stages

(Figure 5.4). Adult *B. violaceus* had a large community component made of genera that contribute to less than 2% of the community, suggesting more species with lower abundances. Dominant taxa associated with the larvae of *B. violaceus*, on the other hand, accounted for 50% of the community (genera that contribute more than 2% relative abundance) (Figure 5.4A).

At a 90% prevalence rate, core taxa were identified to thirteen species present across seawater, adult and larval stages. Eleven core taxa were shared between the larvae and seawater (Figure 5.4D), and eight taxa are shared between adult and larval stages (Figure 5.4C). Four of the eight core taxa between adults and larvae were not identified as larval-water core taxa (*Flavobacteria* sp*., Bacilli* sp*., Ruegeria* sp*.,* and *Roseovarius* sp.), while seven of the thirteen core taxa found in the larval-seawater samples were absent from the adult-larval samples (*Amphritea* sp*., Pseudofulvibacter* sp*., Colwellia* sp*., Pseudoaltermonas* sp*., Tenacibaculum* sp., unidentified sp., and *Vibrio* sp.).

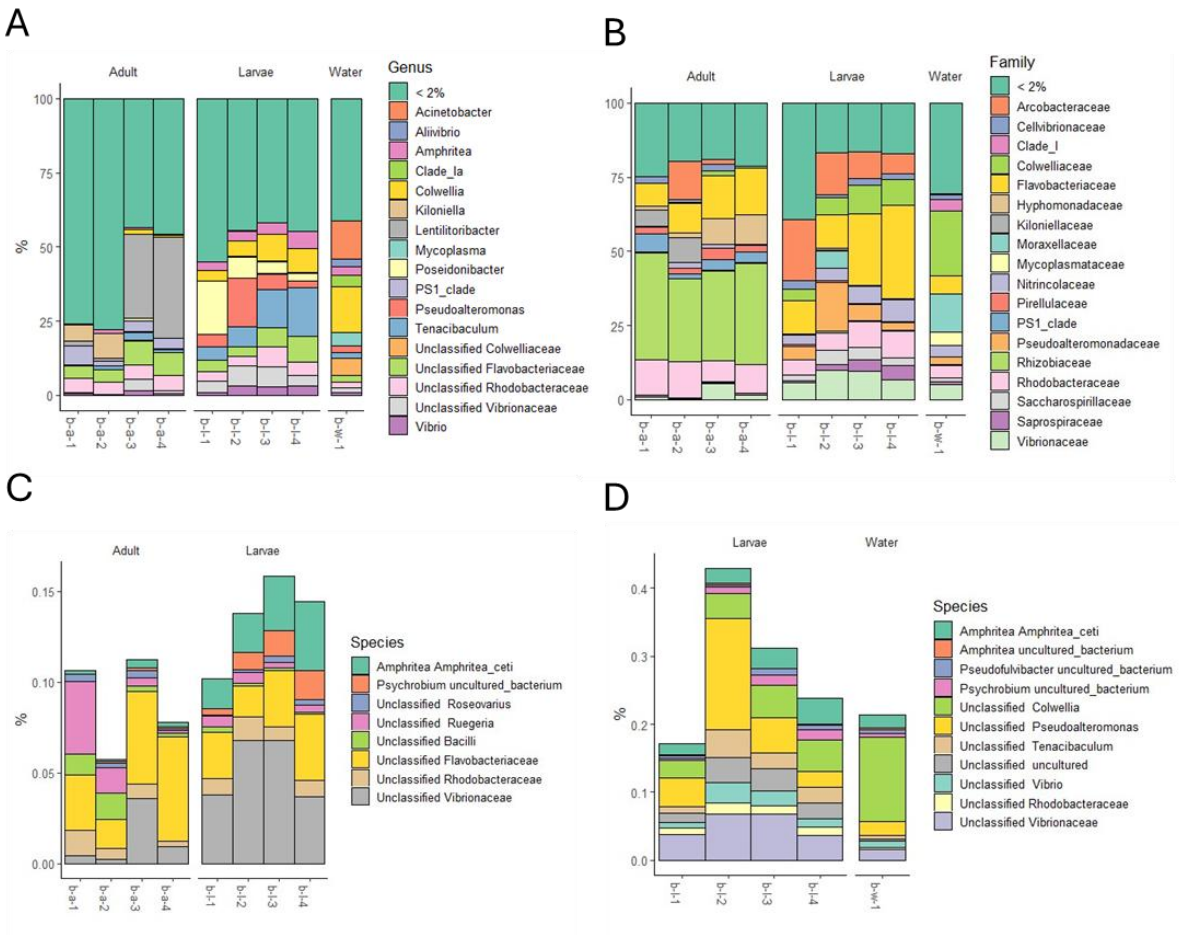


Figure 5.4. Relative abundance community composition of B. violaceus microbiomes at (A) genus and (B) family taxonomic levels. (C) 90% prevalence core community composition between B. violaceus larvae and adults. (D) 90% prevalence community composition between B. violaceus larvae and seawater.

Using the Sourcetracker model, it is estimated that larvae acquire, on average, 24% of their community from adults and 21% from the surrounding seawater, demonstrating a similar influence from both adult and water microbiome transfer (Figure 5.5).

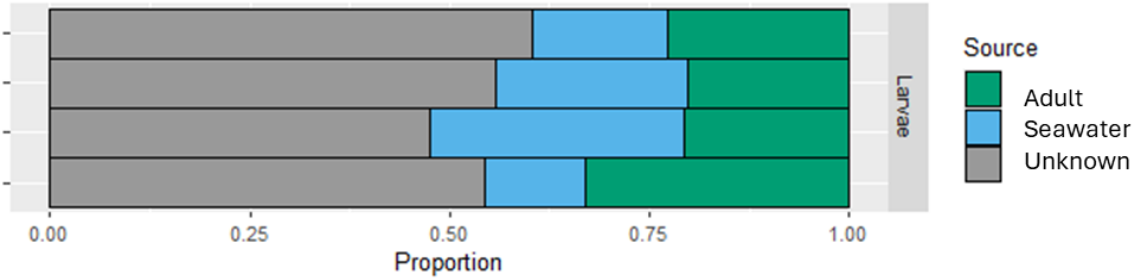


Figure 5.5 Sourcetracker source(water and adult microbiome) proportions found in the B. violaceus larval samples.

ANOSIM analysis identified a significant difference (p=0.02) between the adult and larval microbiomes with an ANOSIM ranking of 0.854 (

Table 5.1), indicating large differences between the two groups (adult vs larvae).

Table 5.1 ANOSIM statistical results between each species' life stages and p-values. The ANOSIM statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. An R-value close to 1.0 suggests dissimilarity between groups, while an R-value close to "0" suggests an even distribution of high and low ranks within and between groups. R values below "0" suggest that dissimilarities are greater within groups than between groups. Only Ciona 'adult vs larvae', 'adult vs water', 'adult vs gamete' and Botrylloides 'adult vs larvae' were significantly different at 95% confidence (p=<0.05). Gamete samples were only collected for the spawning Ciona samples, leaving the brooding species comparisons blank.

	Ciona		***Bugula***		***Amphibalanus***		***Botrylloides***	
Comparisons	anosimR	p.value	anosimR	p.value	anosimR	p.value	anosimR	p.value
Adult.vs.Larvae	1.000	0.028	0.630	0.100	1.000	0.100	0.854	0.023
Adult.vs.Water	0.990	0.029	1.000	0.250	1.000	0.100	1.000	0.200
Larvae.vs.Water	-0.219	0.889	0.333	0.500	0.407	0.100	1.000	0.200
Adult.vs.Gamete	1.000	0.035	-	-	-	-	-	-
Gamete.vs.Larvae	0.352	0.140	-	-	-	-	-	-
Gamete.vs.Water	0.185	0.267	-	-	-	-	-	-

DESeq2 analysis illustrated the differentiation between the two life stages, with *Saccharospirallaceae* sp., NS10 marine group uncultured sp., and *Rubidimonas* sp., differentially abundant in the larval samples compared to the adult.

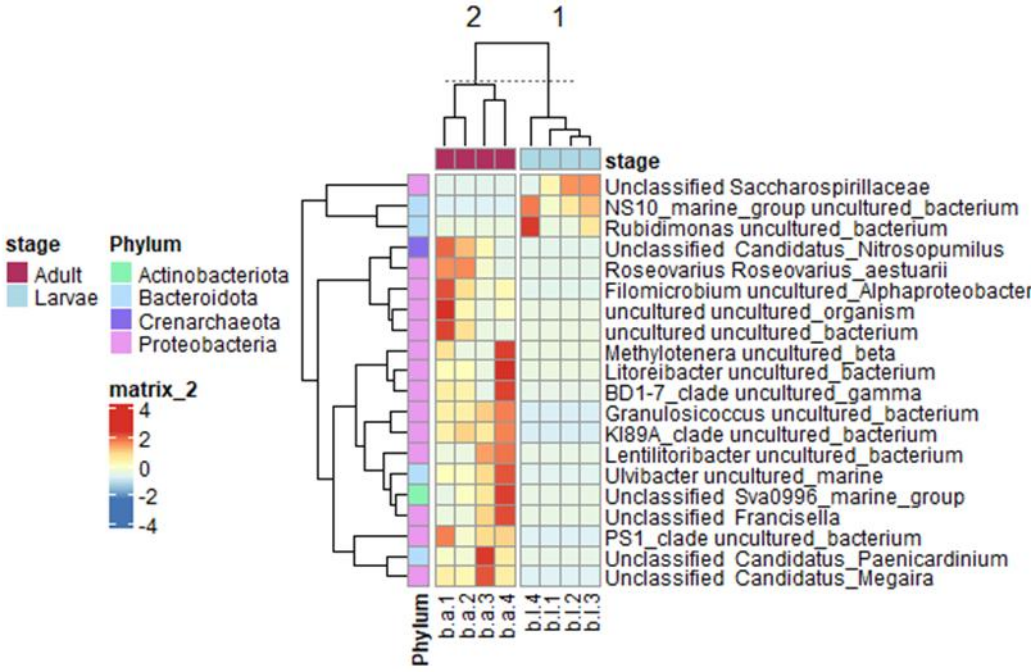


Figure 5.6 DESEQ2 differential abundance between B. violaceus adult and larval microbiomes. Red coded boxes indicate higher differential abundance values, while blue indicates low differential abundance.

5.3.2 Solitary Tunicate: *Ciona intestinalis*

The solitary tunicate, *Ciona intestinalis*, exhibited a large difference between adult and larvae ASVs, with larvae sharing 16.7%% of their total ASVs, and gametes sharing 46% of their ASVs with adults (Figure 5.7A). The total ASV count was low in the gametes (78), larvae (60) and the surrounding water (49) compared to the adult (151), demonstrating relatively low alpha diversity (Figure 5.7B), as well as low relative abundance of unique ASVs (Figure 5.7D). There were distinct differences in beta diversity metrics between adults and their offspring, whereas gametes, larvae and the seawater samples are closely associated (Figure 5.7C).

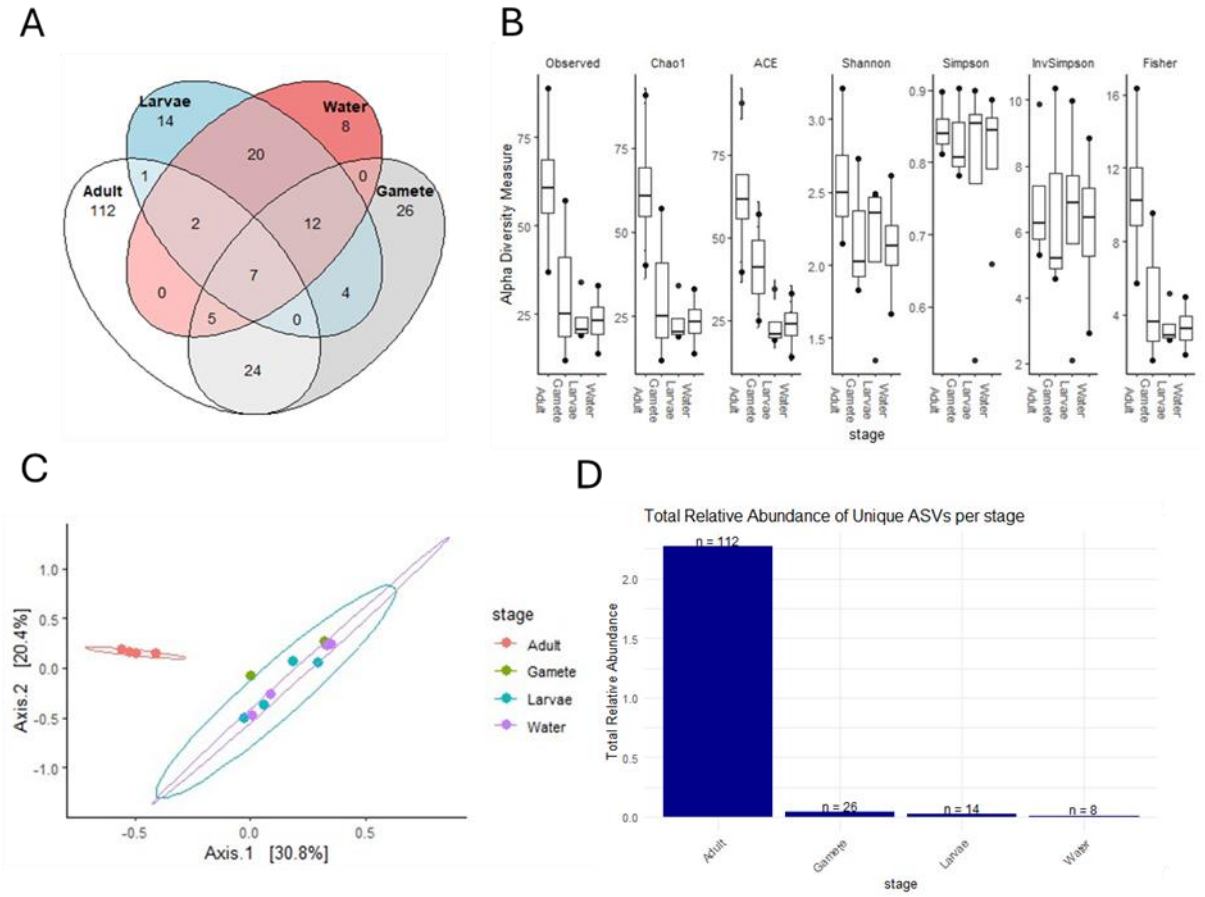


Figure 5.7 (A) Venn diagrams of unique ASVs from C. intestinalis adults, gametes, larvae and the surrounding seawater (B) Alpha diversity and richness from C. intestinalis adults, gametes, larvae and the surrounding seawater. (C) Jaccard beta diversity PCoA plot (D) Total relative abundance of unique ASVs per stage

The community composition of the larvae was dominated by seven taxa at the genus level, comprising over 80% of the community. The larval composition was similar to that of the surrounding seawater, whereas the adult microbial community was dominated by a different set of taxa. The adult microbial composition also had a larger portion of taxa that contribute to less than 2.5% of the total composition (Figure 5.8A and B).

Only one species was present in all adult, gamete, and larval samples: an unclassified *Vibrio* sp. (Figure 5.8C). Eight species formed the core taxa across gametes, larvae, and the surrounding seawater, of which the unclassified *Vibrio* sp. is also present, suggesting the larval microbiome is heavily influenced and obtained by their environment rather than their parents. The importance of seawater as a microbiome source is revealed through the Sourcetracker analysis, which estimates only 2.9% of the gamete microbiome came from the adult stage, compared to 86.8% from the surrounding seawater. This influence increased again at the larval stage, where it is

estimated that larvae acquire, on average, 0.15% of their community from adults and 97.7% from the surrounding seawater (Figure 5.9).

ANOSIM analysis (Table 5.1) demonstrated large R statistics (0.99 – 1), which were found to be significantly different between the adult community and the gametes/larvae communities ($p < 0.05$).

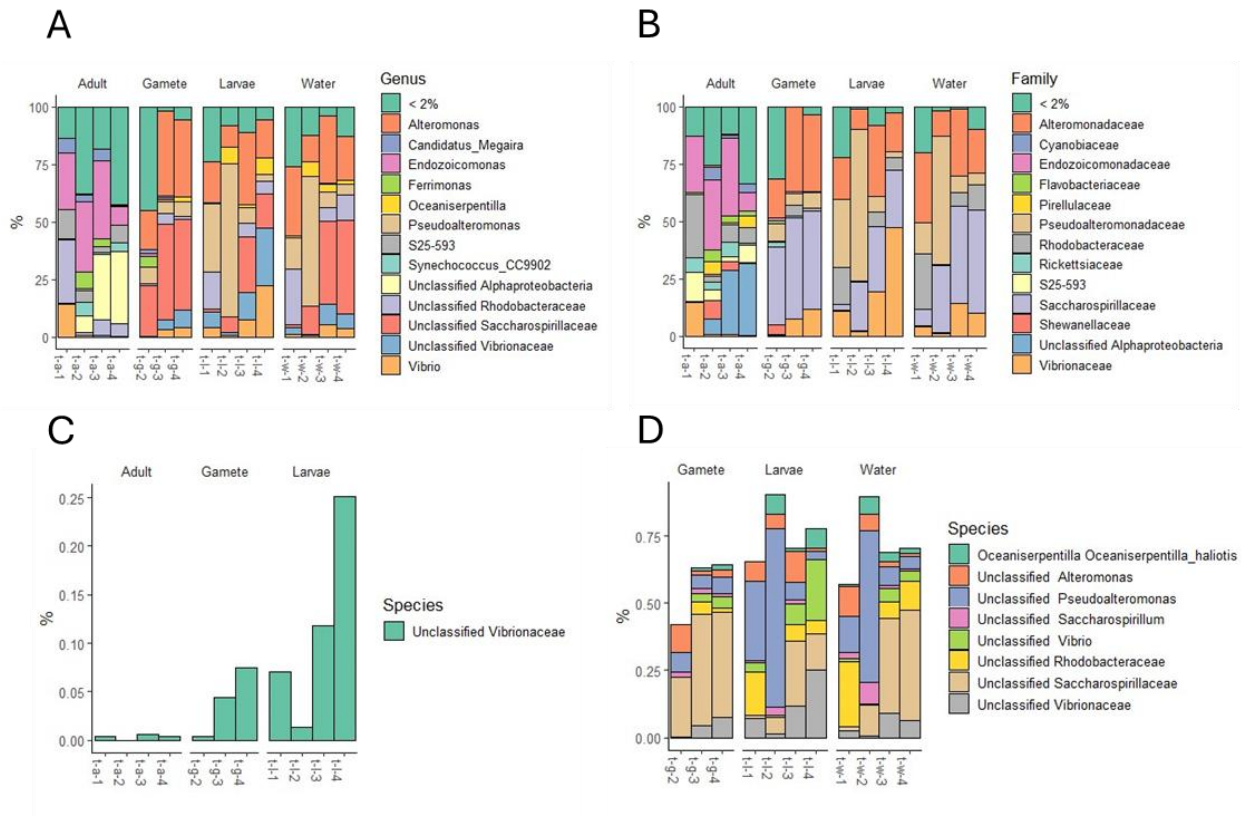


Figure 5.8 Relative abundance community composition of C. intestinalis microbiomes at (A) Genus and (B) Family taxonomic levels. (C) 90% prevalence core community composition between C. intestinalis gametes, larvae and adults. (D) 90% prevalence community composition between C. intestinalis gametes, larvae and seawater.

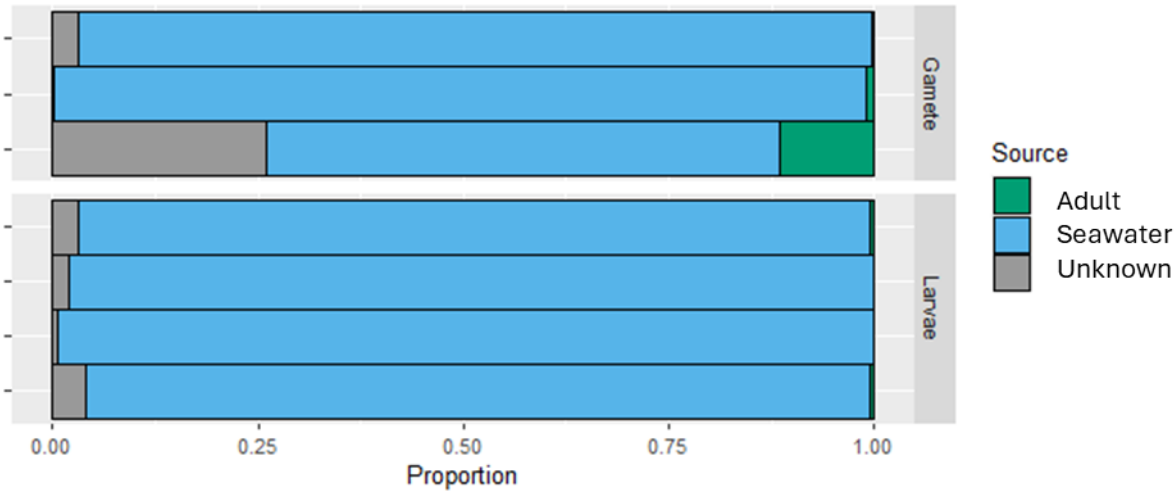
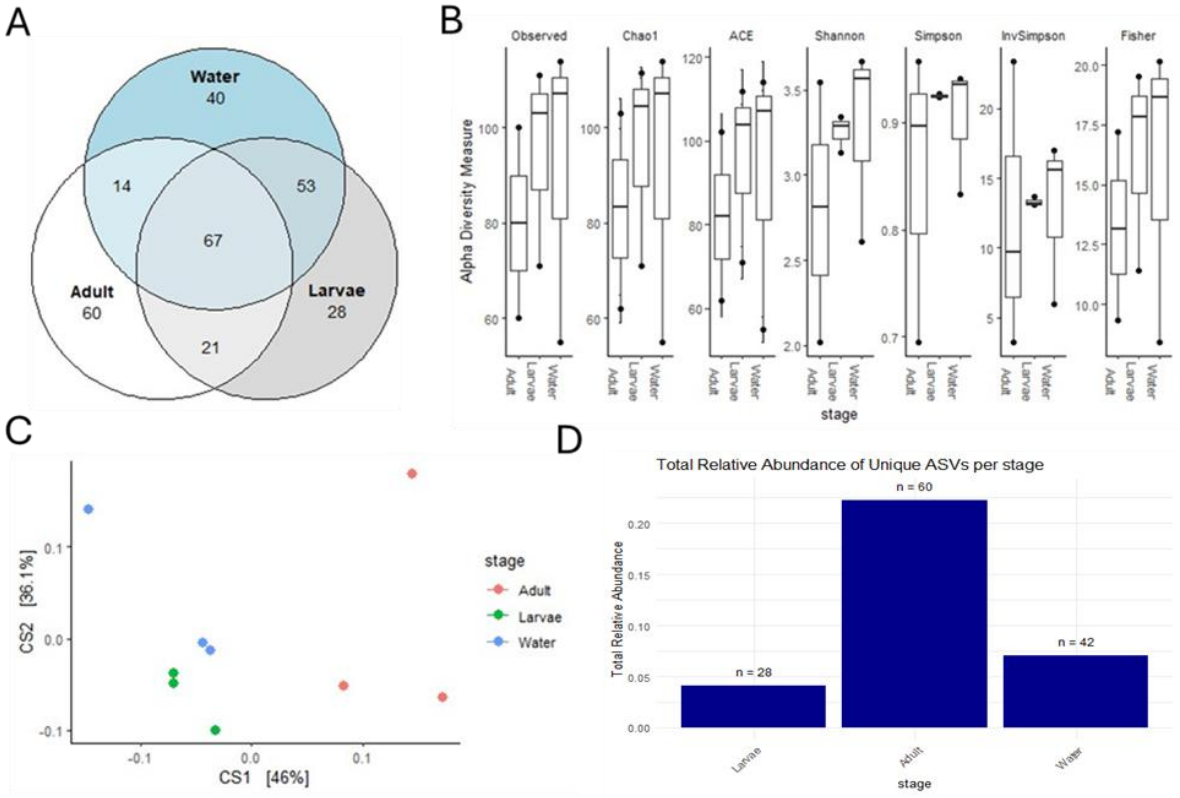


Figure 5.9 Sourcetracker Source(water and adult microbiome) proportions found in the C. intestinalis gametes and larval samples.

5.3.3 Barnacle: *Amphibalanus improvisus*

Amphibalanus improvisus larvae shared a large proportion of their ASVs with the surrounding seawater (71%) compared to those of their parents (52.1%) (Figure 5.10A), with only a small percentage of ASVs unique to each stage (Figure 5.10D). Alpha diversity was higher in the larval and seawater samples compared to the adults (Figure 5.10B). Beta diversity analysis suggests there were distinct differences of composition between the adult, larvae and the seawater (Figure 5.10C), however, there was a closer relationship between the larvae and the seawater communities (Figure 5.10C)



2769

2770 *Figure 5.10 (A) Venn diagrams of unique ASVs from A. improvisus adults, larvae and*
2771 *the surrounding seawater (B) Alpha diversity and richness from A. improvisus adults,*
2772 *larvae and the surrounding seawater. (C) Jaccard beta diversity PCoA plot (D) Relative*
2773 *abudance of unique ASVs per stage*

2774

2775 There were clear differences in the community profiles between the larval samples and
2776 their parents and surrounding seawater, while still sharing some common species
2777 (Figure 5.11A and B) e.g. presence of *Vibrio* and *Rhodobacteracea* across all samples.
2778 There were eight core species identified at 90% prevalence between adults and larvae
2779 (Figure 5.11C), whereas there were 20 core species identified between the larvae and
2780 the seawater (Figure 5.11D).

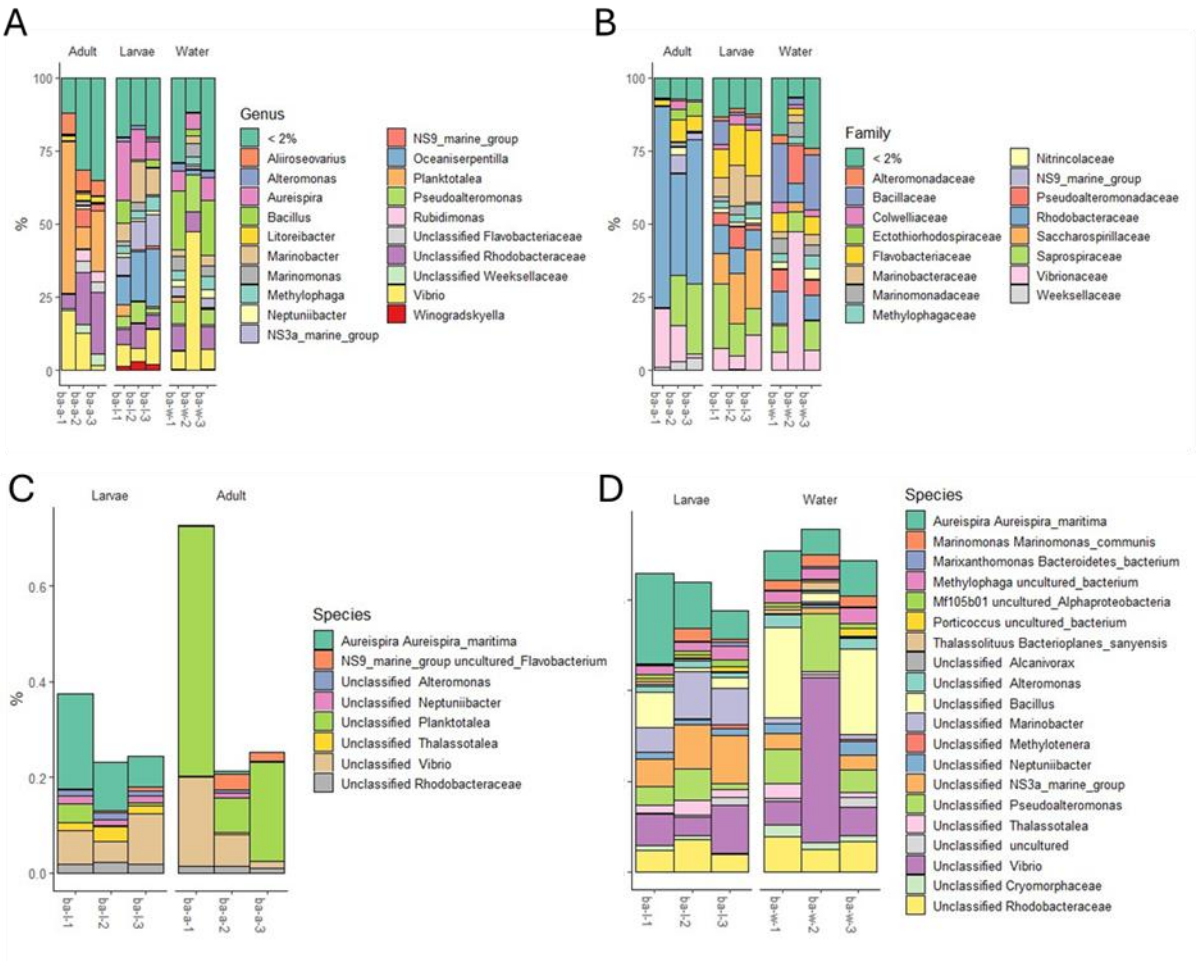


Figure 5.11 Relative abundance of A. improvisus microbial community composition at (A) Genus (B) Family taxonomic levels. (C) 90% prevalence core community composition between larvae and adults. (D) 90% prevalence community composition between larvae and seawater.

Source tracker analysis estimated that the *A. improvisus* larvae gained on average 2.7% of their community from adults and 72.7% from the surrounding seawater (Figure 5.12). This corresponded with the ANOSIM analysis, which gave R values of 1 between adults and larvae, however, were not classed as significant (p =0.1) (Table 5.1).

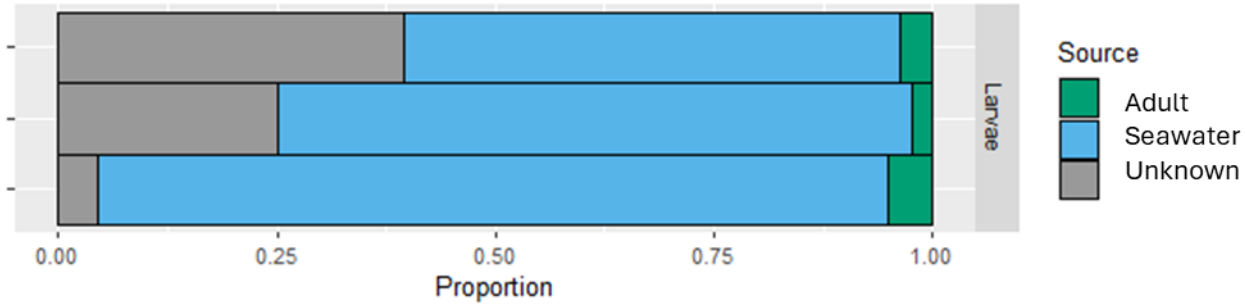


Figure 5.12 Sourcetracker Source(water and adult microbiome) proportions found in the A. improvisus larval microbiome samples.

DESeq2 analysis illustrated the differentially abundant taxa in each stage (adult v larvae, larvae v water) (Figure 5.13). In *A. improvisus* larval samples; *Gammaproteobacteria, Aliikangiella, Spongibacter, Polycyclovorans Solimonas, Cryomorphaceae, Thalassolituss bacterioplanes sanyensis, Kangiella, Marinobacter, Porticoccacea, Alphaproteobacteria* and *Oceaniserpentialla haliotis*, were more abundant in larval samples compared to the adult stage.

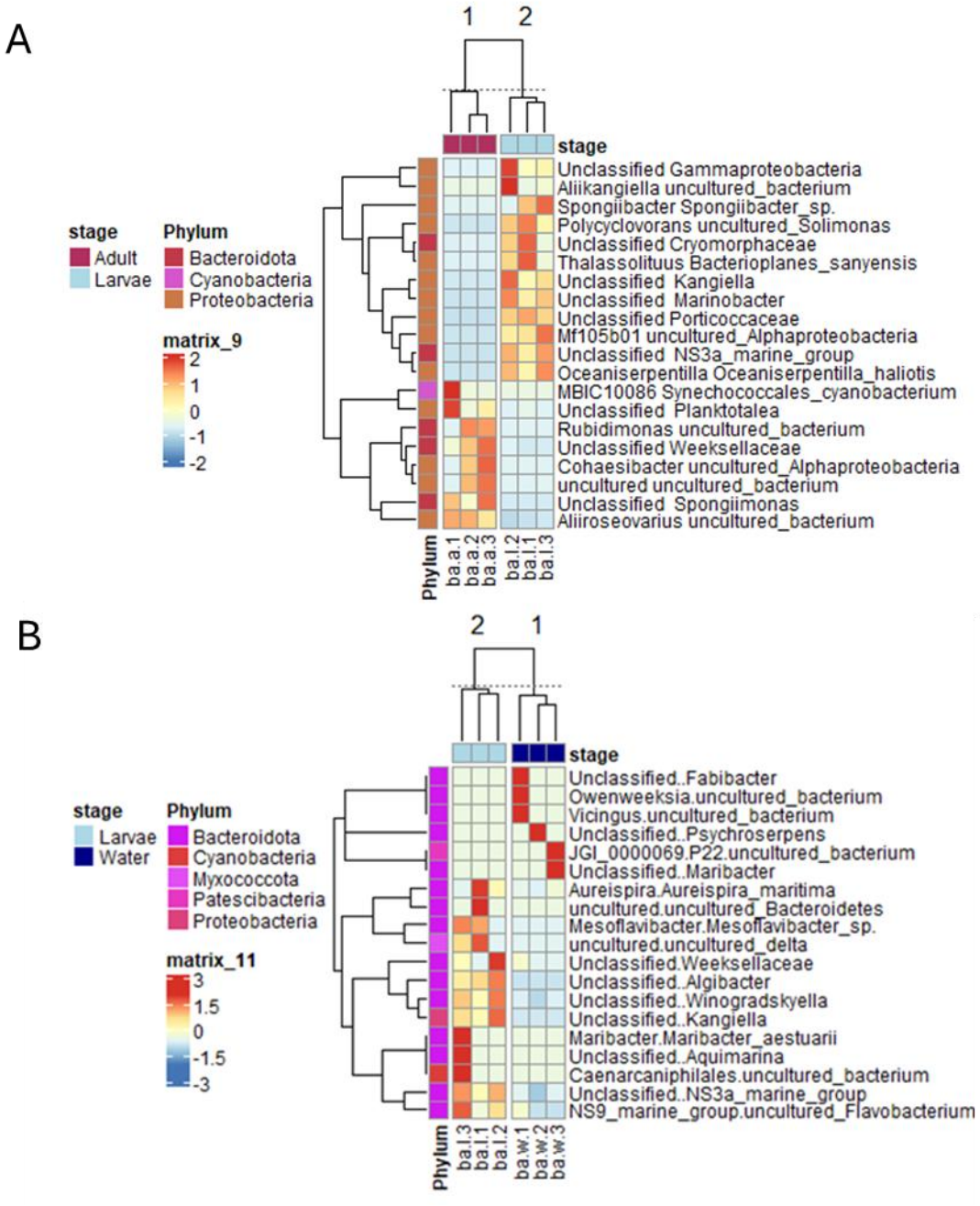


Figure 5.13 A) Differential abundance between A. improvisus adult and larval microbiomes. B) Differential abundance between A. improvisus larvae and seawater microbiomes. Red coded boxes indicate higher differential abundance values, while blue indicates low abundance values.

5.3.4 Bryozoan: *Bugula neritina*

ASV counts were very low for the water sample used in the *B. neritina* experiment. Larvae only shared 8.3% of their ASVs with the seawater, and 50% of their ASVs with their parents. Relative abundance of unique ASVs per stage ranged from 0.6% (larvae) to 1.4% (adults). Even with a relatively large shared ASV community, the alpha

diversity between larvae and adults were very different, with reduced diversity for larvae across all alpha metrics (Figure 5.14B). Beta diversity PCoA (Figure 5.14C) and network analysis (Figure 5.14D) illustrated the dimensional shift between the two stages, although with some overlapping connections between the adult and larvae (Figure 5.14D).

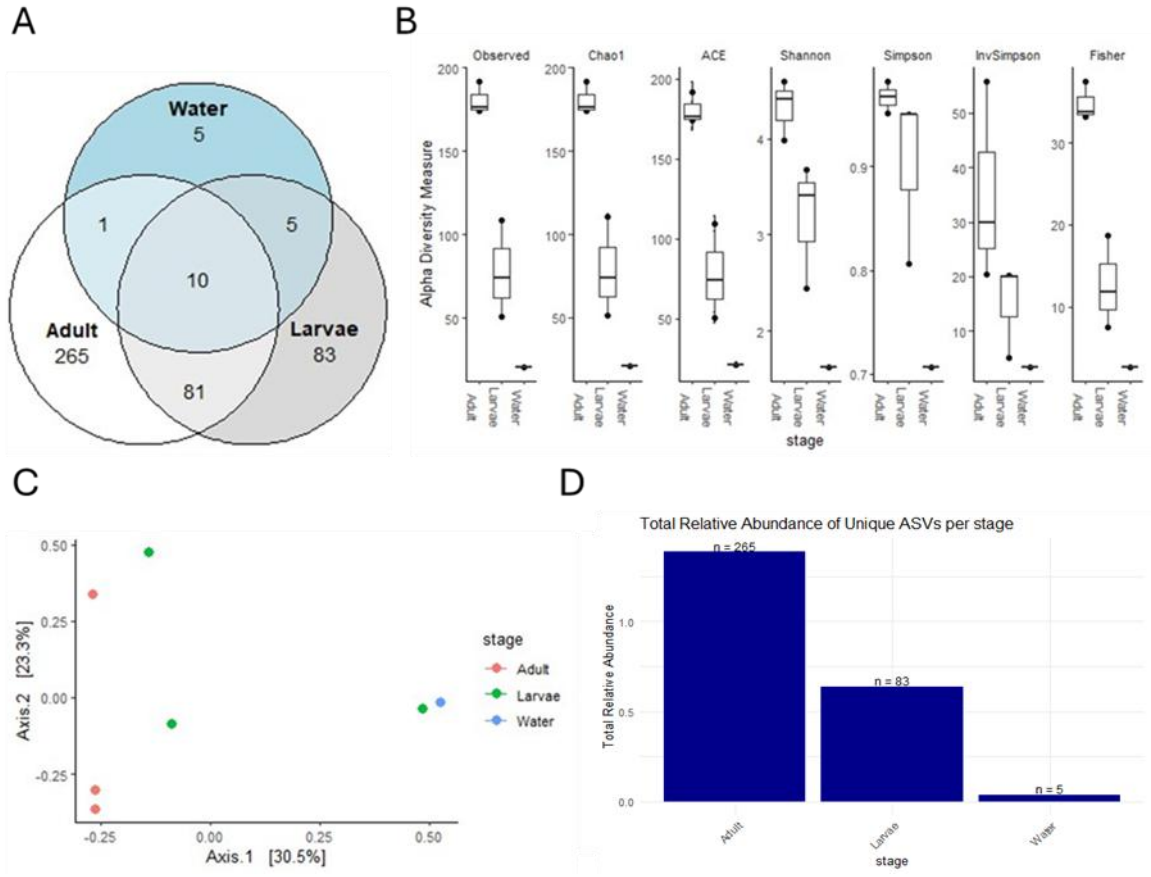


Figure 5.14 (A) Venn diagrams of unique ASVs from B. neritina adults, larvae and the surrounding seawater (B) Alpha diversity and richness from B. neritina adults, larvae and the surrounding seawater. (C) Jaccard beta diversity PCoA plot (D) Relative abundance of unique ASVs per stage

Community composition was also compositionally distinct with different taxa dominating at the different life stages (Figure 5.15A and B). There were six core species identified between the adult and larvae at 90% prevalence (Figure 5.15C), and five species identified as core species between the larvae and seawater (Figure 5.15D). There was a large proportion of *Pseudoalteromonas* in one larval sample, which was also a dominant taxa in the water sample. This large proportion of Pseudoalteromonas correlates with the Sourcetracker results where, in general, the source microbiomes (adult and water) are relatively low except for the one sample

where there were large numbers of *Pseudoalteromonas*. Using Sourcetracker, it was estimated that larvae acquire on average 29.8% of their community from the adults and 17.8% from the surrounding seawater. However, this was likely skewed by one sample which is compositionally different to the other replicates (Figure 5.15). Percentages calculated without the anomaly (sample n-l-2) become 42.2% from the adults and 1.78% from the seawater.

Despite an ANOSIM statistic of 0.63 between *B. neritina* adult and larval samples, the difference was not significant (Table 5.1). ANOSIM between the larvae and seawater samples, however, gave a value of 0.333, suggesting there was more variation within the larvae than there was between the larvae and seawater. This may be due to the anomaly sample (n-l-2) with the unusually high abundance of *Pseudoalteromonas* (Figure 5.15).

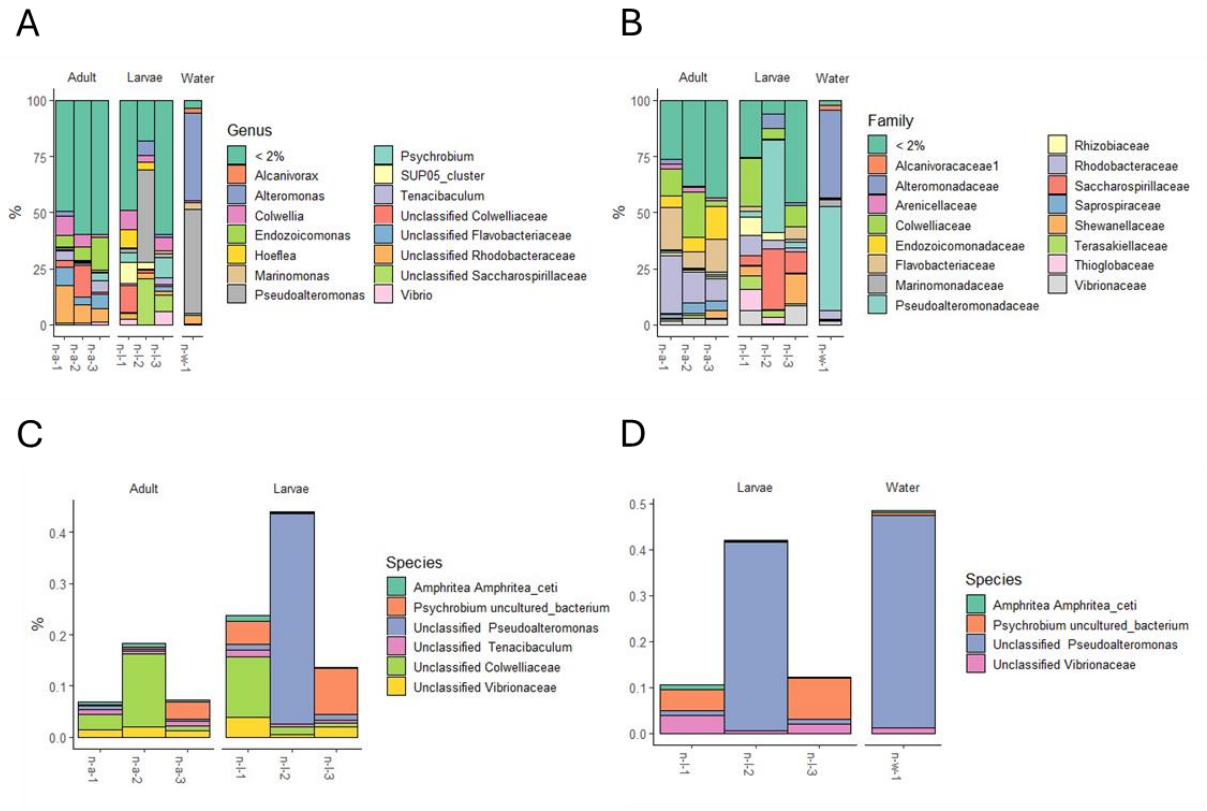


Figure 5.15 Relative abundance of B. neritina microbial community composition at (A) Genus (B) Family taxonomic levels. (C) 90% prevalence core community composition between larvae and adults. (D) 90% prevalence community composition between larvae and seawater.

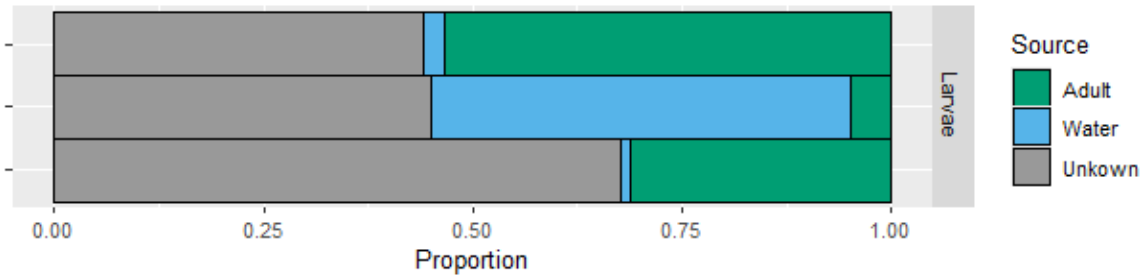


Figure 5.16 Sourcetracker Source (water and adult microbiome) proportions found in the B. neritina larval samples. Green represents adult microbiome source, blue represents seawater microbiome source. Grey represents an unknown source. The sample with the large amounts of water influence is the same one with high amounts of Pseudoaltermonas.

DESeq2 analysis identified six differentially abundant taxa in the larval samples compared to the adults, although not across all replicates (Figure 5.17). *Oceaniserpentilla halitosis*, Uncultured *Alphaproteobacteria*, an unclassified UBA4486 strain, *Hoeflea* sp., an unclassified SUP05 strain., *Sneathiella* sp., and an uncultured Mf105b01 strain were all abundant in the *B. neritina* larval samples.

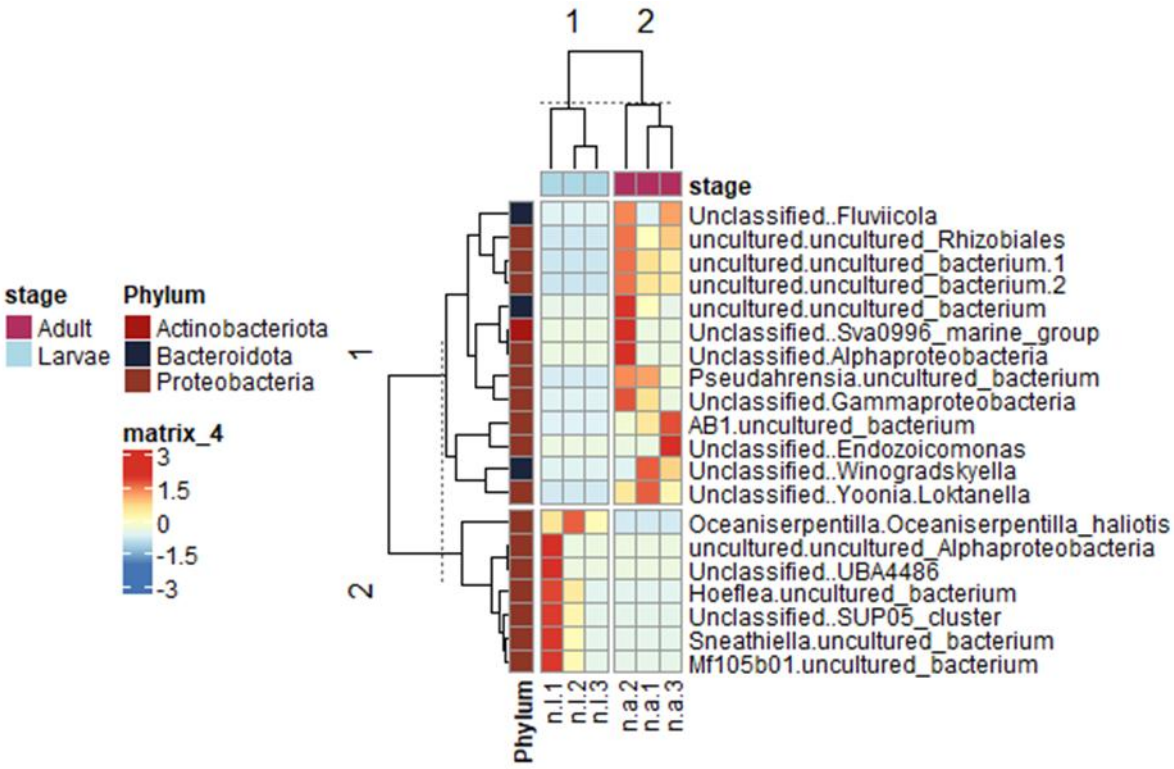


Figure 5.17 Differential abundance between B. neritina adult and larval microbiomes. Red coded boxes indicate higher differential abundance values, while blue indicates low abundance values.

5.4 Discussion

This study assessed the trans-generational transfer of the microbiome of four marine fouling invertebrate species. Microbiome transfer from parent (adult) to offspring (larvae) varied by species, with brooding species generally demonstrating higher rates of vertical transmission compared to the spawning species (*C. intestinalis*). The rate of transfer was quantified using Sourcetracker (Knights *et al.*, 2011), which is the first time this model has been used in this manner, and communities described using diversity metrics and community compositions. Further discussion will focus on each species individually.

5.4.1 *Ciona intestinalis* Microbiome Transfer

Ciona intestinalis spawns unfertilised gametes into the surrounding seawater. These are then fertilised by gametes from other individuals in the water column (Svane and Havenhand, 1993). To assess the transfer of bacterial ASVs from adult to the final larval stage, gametes were extracted in-vitro in order to minimise external influence based on the methods from Yunnie (2009) and Leigh *et al*., (2016). The community composition of both gametes and larvae were significantly distinct from their parents

as demonstrated in the community composition plots (Figure 5.8), beta diversity PCoA and NMDS plots (Figure 5.7) and ANOSIM analysis (Table 5.1). There were 18.6% shared ASVs between adults and gametes, which then reduced down to 4.9% between adults and larvae (Figure 5.7A). However, the Sourcetracker assessment produced much lower contributions. The proportion of microbial species found in the gametes and larval samples averaged only 2.9% and 0.15%, respectively using Sourcetracker (Figure 5.9). The only common taxa that was identified as 'core taxa' between the adults and larvae was *Vibrio* sp. (at a high 90% prevalence rate). However, these were present in the environment too (Figure 5.8C and D). The low transmission rate from parent to larval microbiome in *C. intestinalis* aligns with findings in other *Ciona* sp. microbiome studies, where their microbiomes are strongly related to their environment and geography (Cahill *et al.*, 2016; Utermann, Echelmeyer, *et al.*, 2020). High rates of horizontal microbiome transmission, like in *C. intestinalis* and other invasive tunicates, contribute to their success as invasive species and increase their ability to rapidly acclimate to new localities and habitats (Evans *et al.*, 2018; Dror *et al.*, 2019; Utermann, Blümel, *et al.*, 2020). Although adaptable and diverse, *Ciona* species have still exhibited some stability in their core microbiota across different geographies, likely vertically transmitted which ensures stability and function in host-health (Evans *et al.*, 2018; Dror *et al.*, 2019; Utermann, Blümel, *et al.*, 2020).

In other documented spawning invertebrate species such as oysters, larval microbiomes are partially influenced by their parental microbiome, particularly the maternal egg microbiome. This influence is also partially carried through further development stages (eggs to spat) (Unzueta-Martínez *et al.*, 2022; Scanes *et al.*, 2023). In corals, the photosynthetic dinoflagellate *Symbiodinium* can be transferred inside the egg in some coral species, and has critical benefits including nutritional functions (Baird, Guest and Willis, 2009; Hartmann *et al.*, 2017). Other species may transfer critical symbionts on the outside of the egg where they may later colonise embryos or larvae (Davidson and Stahl, 2008; Davidson, Powell and Stahl, 2010), and are critical for proper development within the egg (Romero and Navarrete, 2006; Sison-Mangus, Mushegian and Ebert, 2015; Mushegian *et al.*, 2018). In other coral species, parental transfer was also assessed to be low, similar to *Ciona* in this study, however, it has been suggested that the adult corals may release bacteria into the environment which their offspring then obtain in early life stages (Ceh, van Keulen and Bourne, 2013). This demonstrates that not all spawning-associated microbiomes are governed

by the same ecological dynamics and, thus, must be assessed on a species-by-species basis. It could be that the sensitivity of 16S rRNA sequencing did not detect particular egg-associated microbes in this study, or the transfer of symbionts to the egg happens upon spawning release, in which case, this was bypassed since in-vitro fertilisation was used. Supplementary shotgun sequencing, which is more sensitive than 16S rRNA sequencing, may help in species detection. Further experimentation could be undertaken to determine the point of microbiome acquisition in *C. intestinalis* eggs and larvae, utilising methods such as microscopy and fluorescent in-situ hybridisation (FISH) of symbionts through the different stages. This has been previously achieved in *B. neritina* larvae (R. M. Woollacott, 1981; Sharp, Davidson and Haygood, 2007).

5.4.2 *Botrylloides violaceus* Microbiome Transfer

Of the species studied, the colonial tunicate *B. violaceus* demonstrated the second largest transfer of microbiome from parent to offspring (24% of their community from adults and 21% from the surrounding seawater) (Figure 5.5). The remaining unknown likely came from either the adult or seawater, however the Bayesian model within Sourcetracker classed it as ‘unknown’. This is likely due to the distinct proportions found at the larval stage and the calculations of Gibbs sampling used in the Bayesian model (Knights *et al.*, 2011). *B. violaceus* adults and larvae shared 18.86% of their total ASVs (Figure 5.3A), which was a similar percentage to *Ciona* (Figure 5.7A), demonstrating that basic percentages of shared ASVs between treatments do not represent the dynamics and influence of common taxa in microbiome transfer. Alpha and beta diversity metrics revealed distinct differences between the adults and larvae (Figure 5.3C and D), as well as the visual community composition plots, which revealed the top dominating taxa at each stage (Figure 5.4A and B). ANOSIM analysis of adults and larvae provided a significant (p=0.02) R Value of 0.854 (Table 5.1), signifying large differences in the community composition between the two groups. Despite these differences, similarities were identified through the core taxa. Taxa that were identified as core species between the *B. violaceus* adults and larvae include *Amphritea ceti*, *Psychrobium* sp., *Roseovarius* sp., *Ruegeria* sp., *Bacilli* sp., *Flavobacteriaceae* sp., *Rhodobacteraceae* sp., and *Vibronacea* sp. (Figure 5.4C). *Amphritea ceti* (Kim *et al.*, 2014) has been poorly studied, but it is part of the phylum *Pseudomonadota* which typically contains a diversity of pathogenic genera such as *Escherichia, Salmonella, Vibrio, Yersinia, Legionella*, and many others (Kersters *et al.*, 2006). Other members

of *Pseudomonadota* found to be core taxa in *B. violaceus* include an unidentified *Vibroanacea*, *Psychrobium*, *Roseovarius, Ruegeria,* and *Rhodobacteracea*. *Flavobacteriacea* were also found to be a core taxon, which also typically contains species that are opportunistic pathogens (McBride, 2014). Another common characteristic of *Pseudomonadota* is the production of anti-bacterial, bacteriolytic agarolytic and algicidal compounds, which have been demonstrated to be beneficial for gametes and larval stages of other marine invertebrates, in particular *Alteromonas* (Ceh, van Keulen and Bourne, 2013; Freire *et al.*, 2019; Nyholm, 2020; Lee *et al.*, 2024) *Rosebacter* sp. *(Rhodobacteraceae*) (Sharp, Distel and Paul, 2012; Freire *et al.*, 2019), and some *Vibrio* species (*V. nereis*) (Freire *et al.*, 2019). *Pseudoalteromonas*, a genus of Gammaproteobacteria closely related to *Alteromonas*, which was a dominant taxon in the *B. violaceus* larval communities in this study, has been frequently related to increased settlement rates in different stony corals (Negri *et al.*, 2001; Tran and Hadfield, 2011) and other invertebrates (Hadfield, 2011). Anti-microbial activity is common among *Alteromonas* and *Pseudoalteromonas* species (Lemos *et al.*, 1991; Shnit-Orland and Kushmaro, 2009; Shnit-Orland, Sivan and Kushmaro, 2012), and thus could be providing beneficial effects as other marine invertebrate species (Shnit-Orland and Kushmaro, 2009; Ceh, van Keulen and Bourne, 2013; Freire *et al.*, 2019). *Rhodobacter* spp., such as *Phaeobacter* spp., also have great potential as probiotic species preventing disease from other bacterial pathogens in organisms such as brine shrimp, oysters, phytoplankton and fish (Prado *et al.*, 2009; Prado, Romalde and Barja, 2010; Karim *et al.*, 2013; Sohn *et al.*, 2016; Sonnenschein *et al.*, 2021; Takyi *et al.*, 2024), as well as inhibiting biofilm formation (Zhao *et al.*, 2016), immunomodulation of larval hosts (evidenced in oyster larvae (Modak and Gomez-Chiarri, 2020)) and disruption of quorum sensing and production of virulence factors (Zhao *et al.*, 2019). The above-listed characteristics and functions could be beneficial for newly hatched larvae and aid in their survivability. However further research would be needed to identify if *Rhodobacter* in particular, is providing this kind of benefit to *B. violaceus* larvae. With supplementary shotgun data, the investigation into specific species may be achieved, compared to some of the limited taxonomic identification of 16S rRNA sequencing. The deeper sequencing depth achieved by shotgun sequence analysis may also elucidate the presence/absence and co-occurrence of virulence genes and anti-microbial resistance (AMR) gene profiles.

Microbiome transfer of specific taxa has been investigated previously in other colonial tunicates. In *Didemnum molle*, it was revealed that specific endosymbionts within the cloacal cavity are acquired by developing embryos, which are carried through release, settlement and metamorphosis to the next generation of adults (Hirose and Fukuda, 2006). Unfortunately, the total proportion of transferrable microbiomes was not assessed through the developmental stages, but it forms the basis for further research.

The large trans-generational transfer of microbiome found in this study raises interesting ecological implications due to the general ecology of *Botrylloides* adults. *Botrylloides* spp. have been described as prolific alien species with a remarkable ability to establish themselves in non-native locations (Bock *et al.*, 2011). This is likely due to their ability to be translocated on ships, the fragmentation of colonies into the environment, and its prolific reproduction cycle (Millar, 1952; Westerman, 2007). *Botrylloides violaceus* is originally native to the Northwest Pacific from northern Japan to southern Korea and northern China, but has been widely introduced to the Northeast Pacific, the Northwest Atlantic, and parts of the Northeast Atlantic (Berman *et al.*, 1992; Ruiz *et al.*, 2000; Lambert and Lambert, 2003; Arenas *et al.*, 2006; Temiz *et al.*, 2024). Within its introduced range, it can displace other fouling organisms, including native and introduced tunicates, bryozoans, barnacles, and mussels through competition for space and food (Schmidt and Warner, 1986; Myers, 1990; Gittenberger and Moons, 2011; Lord, 2017). In all parts of its native and introduced range, *B. violaceus* is more frequently reported from anthropogenic structures than from natural surfaces (Simkanin *et al.*, 2012), therefore its occurrence on anti-fouling coatings likely (Osborne and Poynton, 2019). Although coating-specific microbiomes of *B. violaceus* were not investigated in this study, it is likely that microbiome development is influenced by the settlement substrate (Chapters 3 and 4). Therefore, if colonial fragments are transferred into the environment, and the fact a significant portion of the parental microbiome is transferred to their offspring (Figure 5.5), it is likely that any harboured pathogens, and other undesirable taxa (containing AMR genes) may be spread into the environment. Consequently, a study focusing on *B. violaceus* and other *Botrylloides* species would be an interesting and worthwhile investigation, including assessing a full life cycle of microbiome generational transfer from different anti-fouling treatments and whether mature colonies, with an established microbial community, would re-shift their microbiome if translocated back onto natural substrates.

5.4.3 *Bugula neritina* Microbiome Transfer

For *Bugula neritina*, this study determined there was a 29.8% acquisition of larval microbiomes from parents, and 17.8% from the surrounding seawater (Figure 5.16). The rate from the seawater, however, looks to be skewed and over-inflated due to one of the samples (n-l-2) having far greater similarity to a water sample than the other larval samples, with a large relative abundance of *Pseudoalteromonas* present (Figure 5.15A). Without this anomaly, the transfer rate would be 42.2% from the parents, and 1.7% from the seawater. These data correlate with physical observations in (R. M. Woollacott, 1981) and (Sharp, Davidson and Haygood, 2007) who used microscopy techniques to observe that the larvae of *B. neritina* harboured rod-shaped bacteria in the pallial sinus, a circular invagination of the larval surface, which is effectively sealed from the surrounding seawater (R. M. Woollacott, 1981). Studies have suggested that even during metamorphosis, the bacterial community is reincorporated into the adult community, thus maintaining the trans-generational microbiome (R. M. Woollacott, 1981; Sharp, Davidson and Haygood, 2007). Although there is a heavy influence from the parental microbiome, the larval microbiome is distinct in its own composition when comparing alpha diversity (Figure 5.14B), beta diversity (Figure 5.14C and D), relative abundance of community composition (Figure 5.15A and B) and differential abundance analysis (Figure 5.17). ANOSIM did not identify significant similarities/differences (p=0.1) and suggested a mid-high level of variation between the two communities (R=0.630) (Table 5.1). Some of the similarities were highlighted through the identification of core taxa at a 90% prevalence rate (Figure 5.15C and D). These taxa included: *Amphritea ceti, Psychrobium, Pseudoalteromnonas, Tenacibaculum, Colwelliaceae* and *Vibronaceae*. Similarly to the taxa discussed associated with *B. violaceus*, some of these taxa often display antibacterial and algicidal characteristics, which may be beneficial for the survival of their larvae to adults. *Candidatus Endobugula sertula* has been well-documented for its association with *B. neritina* and its ability to produce bryostatins, which are thought to make the larvae unpalatable to predators (Lopanik, Lindquist and Targett, 2004; Sharp, Davidson and Haygood, 2007). This study only found *E. sertula* in one sample, however this may have been due to low 16S sequencing sensitivity, or quality control steps removing low reads. These studies highlight the benefits of microbiome transfer between generations, and thus, it is likely that other beneficial (or not-so-beneficial) symbionts are transferred.

5.4.4 *Amphibalanus improvisus* Microbiome Transfer

Amphibalanus improvisus shared 52% of its ASVs between adults and larvae, and 71% shared between the water (Figure 5.10A). This is in contrast to the Sourcetracker predictions, which only predicted a transfer of 2.7% from adults (Figure 5.12). Similarly to the other species in this study, the difference between numbers of shared ASVs and community composition illustrates the importance of abundance dynamics when comparing communities. Diversity analysis also demonstrated closer similarities between water and the larvae, although all groups were still distinct from each other (Figure 5.10B-D). Relative abundance composition plots elucidate the presence of similar taxa across the stages but in comparatively different quantities (Figure 5.11A and B). Of the core taxa identified (8) (Figure 5.11C), the relative abundance in the adult and larval samples varied. The abundance of *Vibroneacea* and *Rhodobacteraceae* was similar between the two stages (adult vs larvae), whereas they differed for the remaining core taxa. For example, *Aureispira maritima* had a low abundance in the adults and a far higher abundance in the larvae. Alternatively, *Planktotalea* was abundant in the adult samples, but was much reduced in the larvae. Comparatively, the core taxa between larvae and water were much more similar in abundance and diversity (Figure 5.11D), with 20 core taxa identified (at 90% prevalence), highlighting the heavy influence of the seawater microbiome, while still maintaining its own distinct composition compared to its environment. Using DESeq2 analysis (Figure 5.13), 13 differentially abundant taxa were found in the larvae compared to the surrounding seawater, including: *Aureispira maritima* (a core species in the adults, and previously described as associated with barnacle debris (Hosoya *et al.*, 2007), *Weeksellaceae* sp., *Algibacter* sp., Winogradskyella sp., *Kangiella* sp., and *Maribacter aestuaarii*. The low influence of the parental microbiome may relate to the barnacle's reproductive system and life history traits. The fertilised eggs of *A. improvisus* are brooded in the mantle cavity within an ovisac, for between 7-21 days (Furman and Yule, 1990; Rahmani and Sari, 2009). This ovisac eventually disintegrates, exposing the eggs directly to the circulatory respiratory water current within the barnacle cavity (Walker, 1980). Post-development, free-swimming nauplius larvae are released into the water column, where they feed (from a few days to weeks) before moulting into a non-feeding cyprid larva (Maruzzo *et al.*, 2012; Pansch, Schlegel and Havenhand, 2013). It is likely that barnacle larvae obtain the majority of their microbiome during these planktonic feeding stages as larvae as in other invertebrate species, (Krams *et al.*, 2017; Carrier *et al.*, 2018; Majumder *et al.*, 2019, 2020; Mason

et al., 2020; MacLeod, Dimopoulos and Short, 2021; Vignier *et al.*, 2021; Gohl, LeMoine and Cassone, 2022; Auger *et al.*, 2023), although there is currently no evidence for this specifically for barnacle species.

As evidenced in Chapter 3 in another barnacle species, *S. balanoides*, the microbiomes of newly settled cyprids are heavily influenced by their settling substrate and surrounding microbial communities. It would be interesting to fully assess how much of the parental microbiome makes it through the larval stages and into the metamorphosed juvenile on different substrate microbial communities in all species tested in this study, and whether the absence/presence of certain taxa correlates with reduced fitness, similarly to studies like Vignier *et al.,* (2021) who assessed oyster larvae fitness and microbiome shifts based on dietary exposure.

5.4.5 Trans-Generational Microbiome Assessment and Conclusions

In general, the tested brooder species have the higher potential for microbiome transfer compared to the spawner species tested. Assessment of transfer and influence from the parent or environment requires multiple analyses to gain adequate insight into the dynamics at play. Assessment based purely on shared ASVs gives very little indication of the influence or dominance of the taxa involved, and assessment by comparing alpha diversity metrics, which is used frequently in clinical assessments, also does not reveal compositional differences. Beta diversity metrics such as Bray-Curtis dissimilarity allows composition comparisons with abundance taken into consideration. This, complemented by ANOSIM analysis and Sourcetracker, helps to quantify the similarity of parent and larval microbiomes. Sourcetracker, although with its limitations and alternative design purpose, provided an excellent tool to consider the presence and abundance of ‘source’ microbiomes on the larval or ‘sink’ microbiomes, allowing for a quantitative assessment of transfer - the first time Sourcetracker has been used in this way. With further experimentation of the invertebrate species on different anti-fouling coatings, or any other treatment variable of interest, further assessment on the impact of transfer of non-desirable species (pathogens) or resistance genes of interest could be determined. Other studies have demonstrated impacts on trans-generational transfer in relation to variables such as ocean acidification and climate change (Ceh, van Keulen and Bourne, 2013; Luter *et al.*, 2020; Marangon *et al.*, 2023; Scanes *et al.*, 2023), illustrating that host-symbiont dynamics are susceptible to external influence.

Presence of microbial taxa such as *Alteromonas, Vibrio* and *Pseudoalteromonas*, often indicate antimicrobial properties which can be hugely beneficial, if not critical for larval

survival and development. Investigations like that of Sison-Mangus et al., (2015) and Gil-Turnes et al., (1989) could be recreated with this study's species to determine whether the microbial taxa identified confer benefits (Gil-Turnes and Fenical, 1992; Benkendorff, Davis and Bremner, 2001; Karim *et al.*, 2013; Zhao *et al.*, 2019; Nyholm, 2020; Bunker *et al.*, 2021; Yoon *et al.*, 2022; Takyi *et al.*, 2024).

Additional shotgun sequencing data, that could not be fully analysed within the project timeframe, will be available to support these conclusions post-submission. This will enable basic assessment and detection of virulence genes, pathogenic species and AMR gene profiles, and whether they are transferred from the parents to their offspring. However, further experimentation would be required to compare these from different treatments, e.g. the effect of biocidal coatings on the presence/transfer of these genes. This was the original plan and scope for this study; however, practicalities and limitations of seasonality meant this study was ultimately conducted only as a single trajectory experiment.

As AMR, and the spread of pathogens within the marine environment poses a significant threat to public and environmental health, understanding the dynamics between hosts and symbionts across all life stages is crucial to making evidence-informed decisions and risk assessments. The rates of generational or environmental uptake of microbiomes may also be indicative of invasive species' success in establishing new habitats. It is evident that the types of transferrable symbionts typically have antimicrobial characteristics, highlighting the importance of monitoring and understanding AMR in aquatic environments and marine organisms.

Chapter 6: Discussion

6.1 Summary

The importance of environmental microbiomes and holobionts have been well documented in their importance in driving various environmental and physiological functions, for example, nutrient cycling, larval settlement and resilience. This study aimed to integrate microbiome characterisation and analyses, with anti-fouling risk assessment. The application of these analyses were novel, and provided unique insights into marine invertebrate microbiomes in relation to anti-fouling technology.

The present work demonstrated that marine invertebrate microbiomes are diverse, complex, and dynamic, and they are susceptible to change when directly exposed to biocidal anti-fouling coatings. This study characterised anti-fouling associated microbiomes, from biofilms, invertebrate settlement, established adult fouling communities, through to adult-offspring transfer, and the potential impacts that biocidal anti-fouling coatings may have on those microbiomes. The significance and implications of the thesis findings and future directions of the research are proposed in this chapter.

6.2 Where it All Begins: The Biofilm and its Resistome

Biofilms are incredibly influential microbial communities. For example, they produce settlement cues (Rodriguez *et al.*, 1995; Wieczorek, Clare and Todd, 1995; Hadfield, 2011) and serve as reservoirs of microbial communities for invertebrate microbiome acquisition (chapter 3). This study, among others (von Ammon *et al.*, 2018; Winfield *et al.*, 2018; Catao *et al.*, 2021; Papadatou *et al.*, 2021), found biofilm composition to be coating-specific, exhibiting unique microbial taxa and abundances associated with each anti-fouling coating, as well as overall reduced diversity among the biocidal coatings tested. This was as expected and allowed for the characterisation of the biofilm of which the fouling invertebrates in this study would settle and potentially acquire from. Dominant taxa varied by coating, with each biocidal coating exhibiting its own defined profiles: the copper-only (Coppercoat) coating was dominated by *Marinobacter*, whereas the mixed biocidal coatings (VC Offshore and Trilux) were dominated by *Dasania* and *Cycloclasticus*. The non-biocidal coatings (Ecopower, Silic One and Primer) were all dominated by *Cycloclasticus*, however, Ecopower exhibited more variability in its other dominant taxa, such that its microbial profile more closely resembled the biocidal Trilux than the other non-biocidal coatings (Figure 2.8). Interestingly, the dominant taxa found in these biofilms, such as *Rhodobacteraceae*

and *Cyclobacteriaceae,* have been previously proposed as potential indicators of environmental status (Ramljak *et al.*, 2024).

No significant differences were detected in the relative abundance of potentially pathogenic groups between the biocidal and non-biocidal treatments. However, the Coppercoat coating displayed the highest overall relative abundances of potential pathogens. If flavobacteria were included in the comparison, Ecopower was the next highest group with the most potentially pathogenic bacteria; however, if flavobacteria were not included, Ecopower became the lowest (Figure 2.15). Interestingly, *Vibrio spp.* was absent from the two mixed biocidal coatings, as was the adult *Ciona intestinalis* samples from Chapter 4. Further implications of this are discussed in the section 'Microbiome Acquisition and Anti-Fouling Impacts on Invertebrate Microbiomes'. *Oceanospirillales* on the other hand, was abundant on the biofilm coatings and in the non-biocidal treated *C. intestinalis* samples but was absent from the biocidal treated *C. intestinalis* samples, suggesting that there are further mechanisms at play in regard to microbiome regulation beyond just the absence/presence of microbial taxa.

The profiles of each coating-specific biofilm community were assessed predictive functional models i.e. PICRUSt2 and gene-identified screening using MetaCompare2.0, which assessed for ecological resistome risk (ERR) (i.e. overall mobility of ARGs across a given microbiome) and human health resistome risk (HHRR) (i.e. potential of human pathogens to acquire ARG) (Oh *et al.*, 2018; Afrin Rumi *et al.*, 2024). Predictive Tools like PICRUSt2 allow for glimpses into the functional potential of a model community, however it doesn't give any insight into the specific conditions and drivers that may change the functional characteristics. These type of models often *mischaracterize rare taxa* or underrepresent non-model organisms which are often present in the marine environment (Langille *et al.*, 2013) and thus shouldn't be relied upon for full functional characterisation. It was used in the study to compare between the two methods, as predictive tools are what is available when only 16s data is collected and can still provide an initial screening in key functional shifts based on model communities. The biggest limitation with the model is the available database, as identified in chapter 2. The predictive tool didn't identify polymyxin resistance classes, but this is because the database list didn't have polymyxin related KEGGs available.

Overall, risk scores were higher for the biocidal grown biofilms than the non-biocidal grown biofilms. However, there was variation based on the sequence assembly

method, with the MetaSPAdes approach producing lower ERR scores. This is likely to do with the assembly itself, potentially producing fewer hits against the databases for ARGs, MGEs and pathogen identities. Therefore, it is suggested that any future analysis should use the recommended assembly method IDBA-UD or with MEGAHIT, which produced similar results. Other studies that have adopted this method of assessment also found correlations between risk scores and anthropogenic influence (Chen *et al.*, 2021; Y. Li *et al.*, 2021; Gwenzi *et al.*, 2022; Luo *et al.*, 2023; Mao *et al.*, 2023; Su *et al.*, 2023; Gao *et al.*, 2024; Goh *et al.*, 2024) and it has been a useful tool for environmental monitoring (Pruden *et al.*, 2021; Liguori *et al.*, 2022; Gao *et al.*, 2024; Goh *et al.*, 2024). Environmental monitoring of resistomes is a relatively new field, and a standard procedure for such assessment has yet to be developed. However, the use of MetaCompare and producing risk scores in this way have been cited in 125 published papers since 2020, providing the basis for a promising standard methodology.

Resistome risk refers to the likelihood that genes which are present in environmental resistomes will be introduced into human pathogens (Martínez, Coque and Baquero, 2015; Mao *et al.*, 2023). Although resistance genes have been readily found in various environments, their detection does not necessarily equate to human health risk (Chen *et al.*, 2019). A more indicative measure of risk is the co-occurrence of ARGs, MRGs, MGEs and pathogens as the transfer of these resistance genes is expected to be higher between pathogens and phylogenetically related taxa (Pal *et al.*, 2015; Gupta and Singhal, 2018; Gupta *et al.*, 2018; Chen *et al.*, 2019). MetaCompare2.0 takes three of these factors into consideration. Another pipeline that might provide further insight to supplement with MetaCompare2.0 is Bacmet (Pal *et al.*, 2014), which utilises a curated and extensive database of bacterial genes that are experimentally confirmed to confer resistance to metals and/or antibacterial biocides, which in the context of anti-fouling effects could provide valuable information. Further analysis of any shotgun data related to this thesis will utilise both of these methods to help provide a fuller picture and create a co-occurrence network of these genes. Risk assessment and how it is conducted is however, in need of review. Resistome scores are calculated based on certain criteria, however there is no quantitative thresholds defined to interpret what constitutes “high” risk, and whether this may differ depending on the environmental scenario.

6.3 Microbiome Acquisition and Anti-Fouling Impacts on Invertebrate Microbiomes

The pre-existing biofilm likely plays a role in the microbiome acquisition of settling invertebrates, as illustrated in Chapters 3 and 4. Although host health was not assessed in relation to biocidal exposure and/or microbial associations, it is likely that the absence/presence of particular taxa may mitigate or exacerbate biocidal effects (Rodriguez and Durán, 2020; Pereira, Pilz-Junior and Corção, 2021; Ali *et al.*, 2022; Ashraf, Ahmad and Lu, 2023; Silverstein, Segrè and Bhatnagar, 2023; Diner, Allard and Gilbert, 2024).

In *Semibalanus balanoides*, the microbiome acquisition of settling and juvenile barnacles was very clearly influenced by the coating on which they had settled (Figure 3.6). There were clear similarities between the biofilm compositions (Chapter 2) and the juvenile microbiomes of *S. balanoides* (Chapter 3), which were taken from the same panels in Blyth, but a season apart (the biofilm study was conducted in September 2021 and *S. balanoides* study was conducted in April 2022). Although seasonal variation would likely have influenced the biofilm during the settlement season (Grzegorczyk *et al.*, 2018), there are still comparable taxa. For example, large relative abundances of *Spongiibacteraceae* were found in the VC Offshore, Trilux and Ecopower *S. balanoides* samples and were also found in the Trilux and Ecopower biofilm samples. Similarly, *Cyclobacteriaceae* was a core taxon in the biofilm samples, which was true for most of the *S. balanoides* samples, but it was found in smaller proportions in the Ecopower *S. balanoides* samples. Although the dominant taxa were present in each sample, they are present in varying relative abundances, and the proportion present in the biofilm does not necessarily equate to its abundance or dominance within the invertebrate microbiome. This suggests a layer of host regulation or niche selection. Regardless, the similarities were still apparent, and thus indicate that a large proportion of *S. balanoides* microbiome is obtained from the coating-specific biofilms, and they therefore likely acquire ARGs, MRGs, MGEs and pathogens as identified in Chapter 2. This should be verified using shotgun sequencing which is available for the other invertebrate species studies in chapter 4. As mentioned previously, *Vibrio* spp. was not detected in *C. intestinalis* samples from the two mixed biocidal coatings, which corresponded with the tested biofilm samples. A possible explanation is that the coatings were selecting against *Vibrio* spp. within the *C. intestinalis* microbiome (it could theoretically still be obtained from the surrounding

seawater/environment), or that *Vibrio* spp. was absent and thus not available for microbiome acquisition during *C. intestinalis* settlement. The repercussions of *Vibrio*'s absence are not clear but provide some interesting questions that could be addressed by investigating the function of *Vibrio* spp. within *C. intestinalis* or other invertebrate species. *Vibrio* encompasses some common and unpleasant pathogens, both for humans and marine life, and its controlling its spread is of great interest (Cottingham, Chiavelli and Taylor, 2003; Baker-Austin *et al.*, 2018; Destoumieux-Garzón *et al.*, 2020; Sampaio *et al.*, 2022; Sanches-Fernandes, Sá-Correia and Costa, 2022). Therefore, *Vibrio*'s apparent absence from the biocidal microbiomes highlights an extra potential benefit for biocidal anti-fouling coatings. This, however, did not apply to the other invertebrate species investigated in this study, and its apparent absence, is not evidence of complete absence, but could be a reflection on detection limitations of the methodologies used i.e. 16s primer mismatch or sequencing depth. Other microbial taxa that were found in reduced relative abundances for *C. intestinalis* on the biocidal coatings were *Endoziocomonas* (fish pathogen (Katharios *et al.*, 2015)) and an unclassified Alphaproteobacteria. The former, along with *Vibrio spp.* have been demonstrated to play beneficial roles such as antimicrobial production and holobiont nutrient cycling and, therefore contributes to host health, acclimatisation, and adaptation (Rubiolo, Botana and Martínez, 2019; Pogoreutz *et al.*, 2022; Diwan, Harke and Panche, 2023; Ma *et al.*, 2023; Pogoreutz and Ziegler, 2024). The predicted functional profiles of the *C. intestinalis* microbiomes revealed differences between the copper-only coating and the rest of the coatings, with similar profiles between the other biocidal coatings and the non-biocidal coatings (Figure 4.11). This suggested that in the case of *C. intestinalis*, high copper concentrations can change the functional profiles of its associated microbiome.

The microbiome of *B. neritina,* in comparison, was relatively stable across the non-biocidal treatments but exhibited great variability in its composition in the biocidal treatments, with higher relative abundances of taxa such as *Mycoplasma* and *Amylibacter* (Figure 4.12). *Mycoplasma* as a genus containing human pathogens of interest, would potentially increase the resistome risk score associated with these samples. Interestingly, *B. neritina* microbiomes exhibited greater antibiotic susceptibility on the biocidal coatings, contrary to expectations. It is important to note, though, that only a single concentration of a small selection of antibiotics was tested in this study, based on EUCAST clinical breakpoints. The *B. neritina* microbiome became

more sensitive to ampicillin (beta-lactam) and ciprofloxacin (quinolone) but more resistant to Kanamycin (aminoglycoside). This suggests it is not just a case of increased or decreased resistance, rather the profile of resistance is likely to change with exposure to anti-fouling biocides, and the implications of this would need further investigation. Although the community composition and resistance profiles may differ between the biocidal categories, the predictive functional profiles revealed similar characteristics (Figure 4.16).

6.4 The Spread of Anti-Fouling Biocidal-Induced Microbiomes: Assessing the Risk of Larval Transfer

So far, this study has tracked and assessed the anti-fouling-associated microbiomes from biofilms, through to mature invertebrates (Chapter 2-4). This study also tracked the microbiomes from mature invertebrates to their offspring, including brooding and spawning life histories (chapter 5). It was concluded that the transfer of microbiome was minimal in the spawning species, *C. intestinalis*, but was significant in its contribution to the other brooding species assessed (*Amphibalanus improvisus*, *Bugula neritina* and *Botrylloides violaceus*). SourceTracker was used to make this assessment, having been designed for the purpose of detecting contamination in sequencing protocols (Knights *et al.*, 2011). This novel and alternative use has provided valuable insight into microbiome transfer and quantifying the similarities between the parental and offspring microbiomes, but it does have limitations. Because the process of microbiome transfer is influenced by the surrounding environment, as well as host-related regulation of its microbiome, the proportions of particular taxa from the adult to offspring may be different, and thus may not be detected as 'contamination', hence why there are large proportions of 'unknown' sources across the different species. Other studies that have tried to assess trans-generational microbiome transfer have relied on the comparison of diversity metrics, or counts of shared taxa (Kowallik and Mikheyev, 2021; Unzueta-Martínez *et al.*, 2022; Scanes *et al.*, 2023). This also has limitations, such as large numbers of shared taxa, including from the environment, and in different quantities. A multi-faceted approach is required to truly 'track' the transfer of microbiomes and would likely have to focus on specific microbial taxa. For example *Endobugula* sp. was tracked through *B. neritina* life stages (Sharp, Davidson and Haygood, 2007) using microscopy and in situ hybridisation methods under controlled conditions.

Evidence has been presented that not all of the parental microbiome is transferred and larval microbiomes are otherwise distinct in their composition (Figure 5.4, Figure 5.8, Figure 5.11, Figure 5.15), acquiring their microbiome horizontally (active recruitment of microorganisms from the surrounding seawater) and vertically (intergenerational transfer of core microbes). It would be worthwhile focussing on potential pathogenic species or resistance gene-carrying species that were identified in the parental microbiomes and 'track' if they are also obtained by their offspring. Shotgun sequencing data were collected for the larval transfer experiments, and is pending analysis, which will hopefully provide further insight into species-level identification of pathogens. This will be useful for designing any probes or primers for future experiments. Another angle would be to focus on any 'beneficial' microbiota that may not be transferred if exposed to biocidal coatings, and the potential impact on host health and development. This information is incredibly valuable, as anti-fouling biocides may have wider effects (good or bad) that go beyond primary toxicity of the coatings. Understanding these host-microbe relationships and dynamics would also help in understanding how they change in relation to other external factors, such as pollutants and/or environmental stressors, in evaluating how species may react or adapt to climate change. By deciphering rates of horizontal and vertical transfer, it may also inform on the ability of species to establish in new ecosystems and thus their potential for 'invasiveness' (Evans *et al.*, 2018; Dror *et al.*, 2019; Utermann, Blümel, *et al.*, 2020).

Tracking the transfer of the microbiome between parents and offspring exposed to different conditions/coatings/biocides is important. This was originally the central focus of the study, however, due to logistical challenges of conducting these experiments away from the host-institution, and local environmental challenges (unconfirmed pollution event which caused mass die offs), unfortunately, this goal was not achieved using the wild communities used. Future experimentation could be planned to use cultured species on different coatings within a controlled laboratory environment for potential future study.

6.5 Wider Implications of Anti-Fouling Biocide-Induced Microbiomes

This study utilised pre-made, commercially available anti-fouling paints in the field to obtain 'real life' communities and conditions. This does, however, limit specific conclusions due to the wide range of variables such as: season, use of mixed biocides,

physical texture, hydrophobicity, pollutants (from marinas) and mixed fouling communities. This study identified similarities between coatings, with the 'mixed' biocidal coatings demonstrating more similarities to each other than to the copper-only coating. This could be due to: any extra biocides, the Teflon inside the Trilux and VC Offshore coatings compared to the epoxy base of the Coppercoat, or other physio-chemical properties of the coatings. Ideally, to determine biocide-specific effects or synergistic effects of mixed biocides, the paint formulas should have been kept consistent to rule out other paint property effects. However, the insight gained by using real-life commercial formulations provides a more realistic outcome and based on how biocides are actually used in coatings. The basis of all the biocidal coatings tested are various forms of copper: copper oxide in Coppercoat and VC Offshore, and copper thiocyanate in Trilux. Various biocides have come and gone out of the market due to their negative effects, but copper-based compounds have stayed throughout due to their efficiency and reduced bioaccumulation due to their lipophilic properties (Voulvoulis, Scrimshaw and Lester, 1999). Therefore, the major impacts of biocidal anti-fouling coatings will likely be correlated with microbial responses to copper and/or other metal compounds. Responses include, but are not limited to change in community composition to copper-resistant taxa (Chen *et al.*, 2013; Djaoudi *et al.*, 2022), change in community function (Corcoll *et al.*, 2019), and co-selection of metal and antibiotic resistance (Baker-Austin *et al.*, 2006).

Flach *et al*., (2017) found an increase in antibiotic resistance primarily via chromosomally encoded efflux systems and cross-resistance mechanisms. This was seen through tetracycline resistance, which was said to be a result of co-selection. Increased community tolerance to tetracycline has been detected in copper-contaminated soils (Berg *et al.*, 2010; Fernández-calviño and Bååth, 2013). This highlights that AMR profiles related to biocidal anti-fouling coatings are likely related to the biocides used and the underlying cross-resistance and co-selection mechanisms. In this study, antibiotic resistance was tested within the microbiomes of *C. intestinalis* and *B. neritina*, however, there were no differences between biocidal and non-biocidal treatments. This was likely due to the concentration of tetracycline used. Flach *et al*., (2017) used 20mg/l, whereas this study used 2mg/l. It is likely that the concentration used in this study, which was based on EUCAST clinical breakpoints, was too low to differentiate for higher levels of community 'resistance'.

The risk of having these ‘reservoirs’ of metal and antibiotic-resistant communities is that they can ‘spill out’ into the wider environment, which can impact human health, fisheries and aquaculture and wild populations. Changes in the microbiome can also alter microbiome functionality, which, as discussed in Trevathan-Tackett et al, (2019) can affect ecosystem services, holobiont function and health. Change in the predicted functionality profile was found in the Coppercoat *C. intestinalis* samples, whereas similar profiles were revealed between the other treatments, and within *B. neritina* samples, potentially compensating for the community profile differences.

Considered in the context of the One-Health philosophy, this may ring alarm bells, as microbiomes exposed to biocides and metals such as copper have a higher chance of harbouring AMR or changing pre-existing AMR profiles. The fight against AMR is no longer confined to hospital environments, sewage outputs, agriculture or aquaculture. To effectively combat AMR in the wider environment, a full understanding and surveillance of its prevalence in every environment that humans are exposed to, directly or indirectly, is crucial for proper management and mitigation.

The spread of non-native species and their microbiomes represents a global risk for biodiversity, aquaculture, shipping, and human health (Callow, 1990; Brockerhoff and McLay, 2008; ICES, 2019; Georgiades *et al.*, 2021). To exacerbate the existing risk, marine microbiomes are susceptible to change due to the ever growing threat of environmental and anthropogenic impacts (Thurber *et al.*, 2009; Kraemer, Ramachandran and Perron, 2019; Mittal *et al.*, 2019; Saha *et al.*, 2020; Jurelevicius *et al.*, 2021; Quillaguamán *et al.*, 2021), which can have consequent impacts on environmental functioning (Fan *et al.*, 2013; Wilkins *et al.*, 2019), spread of undesirable microbial species (pathogens) and genes into the surrounding environment (Kraemer, Ramachandran and Perron, 2019; Mittal *et al.*, 2019; Jurelevicius *et al.*, 2021). The mechanisms of spread, whether through vertical or horizontal transfer, combined with host-microbe interactions and regulation (which is often species-specific), represent a complex challenge when trying to assess risk.

6.6 Future Scope

Ultimately, the gap in knowledge that needs to be addressed relates to the lack of surveillance of AMR and pathogens in marine microbiomes. However, there is no standard procedure for this. While there are surveillance methods and legislation set

up for the detection of invasive species and biofouling management (Coutts and Taylor, 2004; Batstone *et al.*, 2009; Dafforn, Lewis and Johnston, 2011; MEPC, 2012, 2023; Floerl *et al.*, 2012; Inglis, Floerl and Woods, 2012; Sinner *et al.*, 2013; Inglis *et al.*, 2013; Pochon *et al.*, 2015; Lewis, 2016; Tait and Inglis, 2016; Georgiades, Growcott and Kluza, 2018; Growcott *et al.*, 2019; ICES, 2019; Georgiades *et al.*, 2020) or AMR in hospitals or wastewater (Pruden *et al.*, 2021; Liguori *et al.*, 2022), it is worthwhile to combine these schemes to include AMR and pathogen surveillance when monitoring the control of fouling species and biosecurity. The importance of AMR surveillance is being highlighted by the WHO, who have seen higher rates of AMR in general since the COVID pandemic (WHO-Europe and ECDC, 2022). However, traditional AMR surveillance utilises culture-based approaches, which are limited to individually culturable bacteria only, which many marine bacteria are not. The use of modern molecular methods allows for wider detection of microbial taxa and AMR genes, as well as assessment of the 'resistome' as a whole. Realistically a combination of methods should be used in the context of One Health assessments (Franklin *et al.*, 2021) and in relation to the spread of invasive species in biofouling management. (Scientific Committee on Emerging and Newly-Identified Health Risks, 2007; Georgiades *et al.*, 2021; Larsson and Flach, 2022).

This study assessed the effects of anti-fouling paints on the microbiomes of fouling species, and further resistome and risk analyses are pending. Further areas of potential study include:

- Co-occurrence network of ARGs, MRGs and virulence genes to unravel the relationships underpinning biocide-related resistance
- Biocide treated trans-generational microbiome transfer of fouling invertebrates and resistome risk assessment to determine the potential of gene spread into the wider environment.
- Identify potential species indicators of increased resistome characteristics within marine microbiomes
- Apply One Health surveillance to marine microbiomes including those associated with anti-fouling biocides.
- Focus on specific biocide-host-microbe interactions, specifically *Vibrio* and *Endozocominas*.

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