Karen Kvindesland

Determining microbiota and antimicrobial resistance (AMR) associated with plastics from different sources

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

-

Karen Kvindesland

Determining microbiota and antimicrobial resistance (AMR) associated with plastics from different sources

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Master in Marine and Coastal Systems

Main supervisor: Nachiket Marathe

Co-supervisor: Maria Angela Serafim

-

Declaration of Authorship of Work

I hereby declare to be the author of this original and unpublished work. All consulted work and respective authors are duly cited in the text and included in the list of references.

Karen Kvindesland

Bergen, Norway

30th September 2024

Copyright on behalf of Karen Kvindesland

Abstract

Plastic wastes and its dissemination into the marine environment pose a serious threat to the marine ecosystems. Plastic surfaces are inhabited by the microbial communities that may be parthenogenic and carry antibiotic resistance genes (ARGs). The aim of the current study was to investigate the differences in microbes present in biofilms on different plastics that are present in different matrices like wastewater (WW) and seawater (SW) using culture-dependent approach. We investigated the biofilm composition of three common plastic polymers; polyamide (PA), polyethylene (PE) and polystyrene (PS), by conducting a controlled laboratory experiment using SW and WW as matrices. Bacterial isolates were obtained from WW and SW as background. Biofilms were successfully established on all polymer types and dominated by *Pseudomonas spp*. Antibiotic susceptibility testing revealed the presence of multidrug resistant bacteria, including potential human pathogen *Klebsiella oxytoca*. Although differences in isolated genera were observed between polymers, the differences were not statistically significant. We isolated multidrug resistant potential pathogens from plastic biofilms, which we were not able to isolate from background matrix previously. Thus, suggesting that plastic surfaces may enrich clinically important antibiotic-resistant bacteria (ARB). This study highlights the role of plastic in dissemination of potential pathogens and ARGs into the receiving aquatic environments and underlines the potential public health risks associated with of plastic pollution in the environment.

Resumo

O desperdício de plástico é uma preocupação ambiental generalizada, com a sua acumulação e disseminação em ambientes aquáticos representando uma séria ameaça para o ecossistema marinho. Este problema é agravado pela colonização da superfície dos plásticos por diversas comunidades microbianas, que podem albergar características patogénicas e transportar genes de resistência a antibióticos (ARGs). Tal colonização microbiana transforma os plásticos em pontos críticos para o enriquecimento e disseminação de bactérias resistentes a antibióticos (ARB), suscitando preocupações tanto a nível ecológico como de saúde pública. O presente estudo teve como objetivo explorar as comunidades microbianas associadas a biofilmes em diferentes tipos de polímeros de plástico sob diversas condições aquáticas, incluindo águas residuais (WW) e água do mar (SW). Um ensaio laboratorial foi desenvolvido para investigar a formação de biofilmes e o subsequente isolamento de microrganismos com resistência a antibióticos.

Três polímeros plásticos comuns foram selecionados para esta investigação: poliamida (PA), polietileno (PE) e poliestireno (PS). Pequenas esferas de cada tipo de polímero foram imersas em matrizes de SW e WW e incubadas sob condições controladas em laboratório, com agitação orbital a 25°C durante cinco semanas. Para avaliar a diversidade microbiana e as potenciais características de resistência, foram isoladas bactérias tanto das matrizes de fundo (WW e SW) como dos biofilmes formados na superfície dos diferentes polímeros. As colónias microbianas foram cultivadas em diferentes meios de cultura, e a seleção foi baseada na diversidade morfológica observada.

As colónias selecionadas foram re-incubadas para obter isolados purificados, que posteriormente foram submetidos a testes de suscetibilidade a antibióticos (AST) para determinar a concentração mínima inibitória (MIC) de vários compostos antibióticos. Este

método permitiu identificar bactérias multirresistentes presentes nos biofilmes. Além disso, o sequenciamento do genoma completo (WGS) foi realizado em 21 isolados bacterianos selecionados com base na resistência ao antibiótico meropenem e/ou na sua diversidade dentro do conjunto de dados global, com o objetivo de elucidar os mecanismos genéticos subjacentes à resistência a antibióticos e à patogenicidade.

Os resultados demonstraram que os biofilmes foram estabelecidos com sucesso em todos os três tipos de polímeros (PA, PE e PS), tanto nas matrizes de SW como de WW. As comunidades microbianas associadas a esses biofilmes foram dominadas por bactérias do género *Pseudomonas*, amplamente reconhecido pela sua versatilidade ecológica e capacidade de adaptação a diversas condições ambientais. A predominância de *Pseudomonas spp.* é provavelmente atribuída à sua elevada capacidade de formar biofilmes. A presença consistente de *Pseudomonas* em diferentes polímeros e matrizes destaca a notável adaptabilidade ecológica deste género e o seu papel potencial na dinâmica dos biofilmes em superfícies plásticas.

Um dos principais achados deste estudo foi a identificação de bactérias multirresistentes nos biofilmes formados nas superfícies plásticas. Entre os isolados, o potencial patógeno humano *Klebsiella oxytoca* destacou-se como uma bactéria multirresistente, capaz de exibir resistência a diversos antibióticos. Esta observação é particularmente preocupante, dada a relevância clínica de *K. oxytoca* e a sua associação com infeções relacionadas com os cuidados de saúde. A presença de resistência antimicrobiana (AMR) nas superfícies plásticas levanta a preocupante possibilidade de que os plásticos possam atuar como vetores para patógenos resistentes a antibióticos, facilitando a sua disseminação em ambientes aquáticos e, potencialmente, entre populações humanas. Notavelmente, várias ARBs isoladas dos biofilmes não foram detetadas nas matrizes de fundo, sugerindo que as superfícies plásticas podem promover o desenvolvimento de determinadas bactérias resistentes a antibióticos e patógenos

clinicamente relevantes. Este efeito de enriquecimento destaca o papel potencial dos plásticos em amplificar os riscos para a saúde pública associados à AMR.

Os resultados também revelaram diferenças na composição microbiana dos biofilmes entre os três tipos de polímeros e as duas matrizes (SW e WW). No entanto, essas diferenças não foram estatisticamente significativas, sugerindo que o impacto global do tipo de polímero e da matriz na composição da comunidade microbiana é relativamente modesto quando comparado com outros fatores ambientais. Ainda assim, a capacidade dos três tipos de polímeros de suportar a formação de biofilmes e albergar AMR destaca a ubiquidade da plastosfera como um potencial perigo ecológico e uma ameaça à saúde humana.

Estes resultados têm implicações significativas, especialmente no contexto global da poluição por plásticos. Com a contínua acumulação de plásticos nos ambientes aquáticos, aumenta a probabilidade de estes atuarem como vetores de bactérias patogénicas e resistentes a antibióticos. A colonização das superfícies plásticas por ARBs e o enriquecimento de ARGs nos biofilmes representam um risco considerável tanto para o meio ambiente como para a saúde pública. A plastosfera constitui um nicho ecológico único que facilita a persistência e disseminação de patógenos e características de resistência, agravando potencialmente os desafios impostos pela AMR à escala global.

Em conclusão, este estudo evidencia o papel crítico da poluição por plásticos nas dinâmicas microbianas e na disseminação da resistência a antibióticos em ambientes aquáticos. Ao demonstrar que os biofilmes formados em PA, PE e PS podem albergar AMR, incluindo patógenos clinicamente relevantes como *K. oxytoca*, reforça-se a necessidade urgente de tratar o desperdício de plástico como uma prioridade de saúde pública e ambiental. Estes resultados contribuem para a crescente base de evidências de que os plásticos não são apenas poluentes inertes, mas participantes ativos em processos ecológicos e de saúde pública. A implementação

de estratégias eficazes para mitigar a poluição por plásticos e os riscos associados é essencial para proteger os ecossistemas marinhos e a saúde humana e animal das ameaças representadas pela AMR. A interseção entre poluição plástica e resistência a antibióticos constitui uma área crítica para investigação futura e desenvolvimento de políticas, com implicações significativas para a gestão ambiental sustentável e a segurança sanitária global.

Acknowledgement

I am grateful to my supervisors Nachiket Marathe and Maria Angela Serafim, in addition to Didrik Hjertanger Grevskott, for the time and effort to help me through my research and provide answers to my doubts.

Furthermore, I would like to extend my gratitude to the team at the microbiology lab Kim and Johanne for their guidance and help in the lab.

I am grateful to IMR and the research council of Norway for facilitating and funding this research and taking me on as a student. Additionally, I would like to thank UAlg and ERASMUS for enabling me to travel for my thesis and providing funding.

Lastly, I would like to thank my fellow students who have been a source of support and knowledge during this process. I would also like to thank my family, mum and dad who have supported me throughout. Thank you to Ragnhild and Gina, and especially Nui, for listening to all my frustrations and offering me their encouragement.

Table of Contents

List of figures and tables

List of Abbreviations

AMR: Antimicrobial Resistance

AMP: Ampicillin

ANOVA: Analysis of Variance

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistant Gene

AST: Antibiotic Susceptibility Testing

AZI: Azithromycin

CFU: Colony Forming Units

CIP: Ciprofloxacin

CHL: Chloraphenicol

COL: Colistin

DDD: Defined Daily Doses

ECC: *E. Coli* Chromagar

ECDC: European Centre for Disease Prevention and Control

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FOT: Cefotaxime

HGT: Horizontal Gene Transfer

IMR: Institute of Marine Research

MALTI-TOF-MS: Matrix Assisted Laser Desorption – Time of Flight Mass Spectrometry

MA: Marine Agar

MAC: MacConkey Agar

MERO: Meropenem

MHA: Mueller Hinton Agar

MHB: Mueller Hinton Broth

MIC: Minimum Inhibitory Concentration

MP: Microplastic

mRNA: Messenger RNA

PA: Polyamide

PBS: Phosphate Buffer Saline

PE: Polyethylene

PP: Polypropylene

PS: Polystyrene

SMX: Sulfamethoxazole

TAZ: Ceftazidime

THF: Tetrahydrofolate

TET: Tetracycline

TGC: Tigecycline

TMP: Trimethoprim

SW: Seawater

WGS: Whole Genome Sequencing

WW: Wastewater

WWTP: Wastewater Treatment Plant

Introduction

Plastic

Plastic is a man-made combination of synthetic and semi-synthetic materials made up of large molecules, i.e. polymers, often fused with harmful additives during the manufacturing process (Hermabessiere *et al.*, 2017; Atanasova *et al.*, 2021; Lear *et al.*, 2021). The overwhelming majority of plastics are derived from non-renewable fossil fuel sources such as oil and natural gas (Atanasova *et al.*, 2021; Lear *et al.*, 2021). However, some may be made up by sources considered bio-based and renewable, such as corn starch and sugar beet (Lear *et al.*, 2021).

After World War I, development in technology improved which led to improved development of plastics (Lear *et al.*, 2021). Since then, plastics became important in industry and its production quadrupled during World War II (Worm *et al.*, 2017). The first commercially produced plastics were made in the early 1900s (Lear *et al.*, 2021), but since the wars, they have become ubiquitous in a variety of household products ranging from food packaging, personal hygiene products, medical supplies, industrial processes, etc (Worm *et al.*, 2017). They have intrinsically become a necessity to modern society.

As such, there has been a rampant increase in plastic production since the 1950s @. In 2021, an estimated total of 8300 tonnes of virgin plastic was produced annually (Yildizhan, 2021). In 2017, it was estimated that only 30% of all plastic ever produced was in use at the time (Geyer *et al.*, 2017) and by 2030, 53 million tonnes of plastic waste is estimated to be produced (Borrelle *et al.*, 2020). This total is predominantly comprised of the production of polypropylene (PP) and polyethylene (PE), two non-biodegradable polymer types deriving from fossil fuels (Harding *et al.*, 2007; Schwarz *et al.*, 2019). Additionally, polystyrene (PS) and polyamide (PA) are among the commonly produced plastics contributing to this massive waste stream (Vasanthan, 2009; Hirschberg and Rodrigue, 2023). PAs are characterised by polymers bonded by amide and colloquially known as nylon and has a wide range of uses such as in textiles, carpets and tires (Vasanthan, 2009) and can be bio, or fossil fuel based (Salazar *et al.*, 2014). PS also encompasses a wide range of uses, including construction and insulation, packaging foam, food containers and disposable cups and cutlery and is petroleum based (Dybka-Stępień *et al.*, 2021). PS and PA can be recycled but it is a difficult process leading to the majority of wastes ending up in landfills (Dybka-Stępień *et al.*, 2021; Hirschberg and Rodrigue, 2023).

Less than 1% of plastics produced are derived from bio-sources, and they do not necessarily biodegrade or break down at a faster rate (Weinstein *et al.*, 2020; Lear *et al.*, 2021). This constitutes a huge problem for the disposal of plastics. Given the exponential growth in plastic production, the waste treatment management of the excessive load of polymers has not kept up (MacLeod *et al.*, 2021). On a global scale as of 2017, only an estimated 9% of plastics are recycled, 2% incinerated, and the remaining 79% goes to landfills or enters the natural environment (Geyer *et al.*, 2017).

Plastics in the natural environment

The accumulation and pollution of plastic wastes in the natural environment is colossal. Between 9 and 25 million tonnes of plastic is estimated to reach the terrestrial environment on an annual basis, followed by up to 23 million tonnes reaching the marine environment (MacLeod *et al.*, 2021). Larger plastic items will weather and break down into smaller plastic items over time after its entered the natural environment (Lechthaler *et al.*, 2020). Moreover, erosion of plastic items in use, such as tires and ropes, also release small plastic fragments (Ostle *et al.*, 2019). This consists of different size classes of plastics known as macro- micro- and nano plastics. Debris that measure over 5 mm in size are recognized as macro plastic, those less than 5mm are considered microplastics (MP) and those less than 1 mm are categorised as nano plastics (Worm *et al.*, 2017; Ostle *et al.*, 2019).

As previously stated, the marine environment is not exempt from plastic pollution either. Due to global ocean circulation and gyres, the distribution of plastics in the marine space is extensive and has been documented in all oceans and seas on the planet (Lartaud *et al.*, 2020). Plastic polymers reach the marine environment either directly through littering, especially of fishing gear (Thushari and Senevirathna, 2020), or indirectly through failed filtering of wastewater treatment plants (WWTP) (Thushari and Senevirathna, 2020; Tang *et al.*, 2023). Land and ocean-based sources are both important to the accumulation of plastics in marine systems. It is estimated that land-based sources, such as freshwater input, residential and domestic activities, tourism and harbour operations constitute 75% of plastic pollution to marine environment (Thushari and Senevirathna, 2020).

Furthermore, sea ice is constantly interacting with the marine environment and has been described as a source, sink and medium of transport for synthetic polymers (Kanhai *et al.*, 2020). As such, it can trap MPs from the ocean during ice formation or release MPs into the marine space during melting. This can be permanent or seasonal. However, ice polluted with

MPs is not only limited to sea level. MPs have been reported in snow packed glaciers on mountain peaks, such as Mount Everest (Napper *et al.*, 2020). These fragments are assumed to derive mainly from climbers clothing and equipment. The noteworthy outcome, in conjunction with global climate change and consequently retreating glaciers, is a novel pathway of MPs into the marine environment (Beard *et al.*, 2022a). There is an ongoing trend in the Northern hemisphere that the overwhelming majority of glaciers are retreating, and minority are advancing due to climate change (Dixit *et al.*, 2021; Kochtitzky and Copland, 2022). As such, the influence of sea ice as a vector for MPs is expected to change (Kanhai *et al.*, 2020) and likely impact the ocean negatively. Ultimately the introduction of plastics to marine ecosystems poses a variety of ecological and environmental threats.

Impacts of plastic pollution to marine life

As a result of widespread plastic pollution in the marine space, marine organisms have become susceptible to complications by it. Ingestion and entanglement of plastics by marine organisms is not uncommon and has been documented across several marine taxa including seabirds, marine mammals, sea turtles, fish, and invertebrates (Kühn and van Franeker, 2020). Entanglement mainly pertains to the pollution of fishing gear such as nets and ropes (Thushari and Senevirathna, 2020). Both entanglement and ingestion of plastic fragments can be lethal to marine organisms either directly by injury consequent to the incident or indirectly by a reduction in ability to perform biological responses such as prey hunting and ingestion, sexual production abilities, loss of mobility and sensitivity or response mechanisms (Thushari and Senevirathna, 2020). As such, the phenomena of entanglement and ingestion composes a great threat to the ecosystem by impacting a wide range of ecosystem functions and services.

Furthermore, plastic is considered a major disturber and stressor to coral reef communities (Pinheiro *et al.*, 2023). Large plastic debris items can cover and suffocate colonies through inhibition of light exposure and toxin release (Lamb *et al.*, 2018), whereas smaller items can be ingested either by corals themselves, or bioaccumulated as zooplankton ingests MPs causing physiological disfunction (Richards and Beger, 2011; Lartaud *et al.*, 2020). Plastic wastes are strongly linked with the occurrence and breakout of diseases in reef communities, such as white syndromes caused by opportunistic pathogenic genus *Vibrio spp.* (Lamb *et al.*, 2018). When in contact with plastic wastes, the likelihood of reef communities obtaining disease increases with 85 percentage points (Lamb *et al.*, 2018). This is either due to enervation or physical injuries which may or may not thereafter be exposed to pathogens (Lamb *et al.*, 2018) due to the

potentially pathogenetic characteristics of plastic surfaces (Lamb *et al.*, 2018; Marathe and Bank, 2022). The exterior of plastic particles is inhabited by unique microbial communities and is referred to as the plastisphere (Marathe and Bank, 2022).

Plastisphere

The phenomena of adhesion and colonisation of microbiota on plastic surfaces has long been reported. First coined by Zettler et al., (2013), the plastisphere is a collective term describing distinct microbial communities affixed to plastic debris compared to other microbial communities in the immediate surrounding environment (Marathe and Bank, 2022). One study explains this by the leachate from plastics being more bioavailable for these organisms as there is more carbon present compared to organic compounds (Sheridan *et al.*, 2022). Though, there is also genetic evidence that some microbes utilize plastic for energy (Zettler *et al.*, 2013). The plastisphere is composed by hetero- and autotrophs, predators, and symbionts Zettler *et al.*, 2013).

In a world of increasing plastic production and subsequently increasing disposability and pollution, plastic particles have become an available biosphere to inhabit. Due to the long residency time of plastics in the environment caused by their slow rate of decomposition, these ecosystems persist for a long time (Thushari and Senevirathna, 2020; Marathe and Bank, 2022). Furthermore, this combined with plastic debris' high ability of dispersal in the oceans is a cause of concern should these microbial community be pathogenic in nature. There are several reported incidents of opportunistic pathogens associated with plastic surfaces @. Radisic et al. (2020) found opportunistic human pathogens *Acinetobacter beijerinckii* and *Morganella morganii*, the latter being multidrug resistant.

Antibiotics

Antibiotics are naturally occurring compounds produced by microorganisms which have been used for treating a variety of ailments for centauries (Larsson, 2014). The breakthrough of synthetic antibiotics dates back to the 1910s (Hutchings *et al.*, 2019) but it was the discovery of penicillin in 1928 by Alexander Flemming that revolutionized medical treatment of bacterial infections (Alduina, 2020). Now, its overuse has become a global health concern as most present-day prescriptions and sales are unnecessary (Cook and Wright, 2022). Even with the

dismissal of antibiotics in human clinical use, misuse is still a pressing issue due to its overuse in agriculture to promote growth and prevent disease amongst livestock (Chang *et al.*, 2015).

Antibiotic resistance

By nature, the existence of antibiotic properties will create selection pressure among microbial communities favouring antimicrobial resistance (AMR) (Kolář *et al.*, 2001; MacGowan and Macnaughton, 2017). These are genetic expressions that can be mutated or acquired (Aminov and Mackie, 2007; MacGowan and Macnaughton, 2017). This is the fundamental driver of the evolution of antibiotic resistant bacteria (ARB) (Kolář *et al.*, 2001). Selection pressure may manifest itself in Horizontal Gene Transfer (HGT) (Frieri *et al.*, 2017). HGT is the transfer of genetic material between organisms not parentally related (Goldenfeld and Woese, 2007; Soucy *et al.*, 2015). However, the onset of mass production of antibiotics and subsequent increase in prescriptions and usage has caused an exponential increase in reported instances of AMR, and new antibiotics are not developed at an according pace (McCoy *et al.*, 2011; Nathan *et al.*, 2014). The irreversible damage of antibiotic overuse is becoming fatal as infections once treatable with antibiotics are now beyond remedy (MacGowan and Macnaughton, 2017). In fact, current projections suggest it could lead to 10 million deaths annually worldwide by 2050 .

The molecular mechanisms of antibiotics ultimately inhibit biosynthesis in the bacteria leading to lethality (Sullivan *et al.*, 2020). One way is by targeting the protein synthesis inhibitors by binding to specific sites on the ribosome and disrupting the translation of messenger RNA (mRNA) into proteins, ultimately preventing the bacteria producing essential proteins necessary for survival and growth (McCoy *et al.*, 2011). DNA synthesis disruption is another mechanism used by antibiotics, either by overexpressing certain genes, so that ATP becomes reduced (Pontes and Groisman, 2020), or enzymatic modification preventing DNA to twist (Nicolaou and Rigol, 2018; Santos and Lamers, 2020). In such cases, resistance is displayed by membrane pumps actively discarding the antibiotics, enzymatic activities and mutations of target site (Santos and Lamers, 2020).

Another way in which antibiotics act against bacteria is by disrupting membrane synthesis or the integrity of the cell wall (Vranakis *et al.*, 2014). This could be by interfering with the synthesis of peptidoglycan, a key component of bacterial cell walls, making it more susceptible to osmotic pressure which could lead to cell lysis. β-Lactams, including penicillin, ampicillin (AMP) and meropenem (MERO), are a common group of antibiotics that work as such

(Hellinger and Brewer, 1999; Sharma *et al.*, 2013). Bacteria resistant to β-Lactams have adopted different strategies to protect themselves, including altering penicillin binding proteins, efflux pumps which remove the antibiotic from the periplasm, or the production of enzymes that hydrolyse the rings of β-Lactams, such as β-lactamases (Vranakis *et al.*, 2014). However, some antibiotics such as cefotaxime (FOT), uses β-Lactam rings to destruct cell walls and works effectively against many Gram-negative species and adequately against some Gram-positive species

.

Antibiotics in the natural environment

Though antibiotic molecules are naturally produced by some microbes, the mass production in the 20th century has allowed for synthetic antibiotic pollution in the natural environment (Larsson, 2014). Its dissemination to the environment can take place from production to after usage. It can be introduced by discharges during manufacturing, disposal of medicine and excretion (Larsson, 2014). High concentrations have been reported in industrial and domestic effluents (Larsson, 2014). Agricultural runoff waters have also been reported to contain substantial concentrations of antibiotics though which vary with the presence of vegetation (Dolliver and Gupta, 2008).

Antibiotic resistance and the plastisphere

Areas abundant in plastic, especially MP pollution, are also likely to be rich in antibiotics as they share many of the same pathways into the natural environment (figure 2). Domestic and clinical wastes contain plastics that can be fragmented and become secondary microplastics, and antibiotics are part of this category through excretion (Stevenson *et al.*, 2024). Treated wastewater (WW) used for irrigation, introduces MPs to ground water, which may also be filled with antibiotic residues from human and agricultural use (Ofori *et al.*, 2021). Plastics are utilised in various sectors in the food industry such as agriculture for mulching practices and in aquaculture (Eckert *et al.*, 2018; Stevenson *et al.*, 2024). This is in addition to antimicrobials excessive usage in veterinary medicine and prophylactic usage, especially in aquaculture (Eckert *et al.*, 2018; Stevenson *et al.*, 2024). The cryosphere is also known to sequester MPs (Chubarenko *et al.*, 2023) and ARGs have been discovered in Arctic permafrost (Mindlin *et*

al., 2008; Haan and Drown, 2021). Lastly, waste streams including WW, livestock manure and solid waste all contribute to the pollution of plastics and antimicrobials in terms of AMR and ARB (Stevenson *et al.*, 2024).

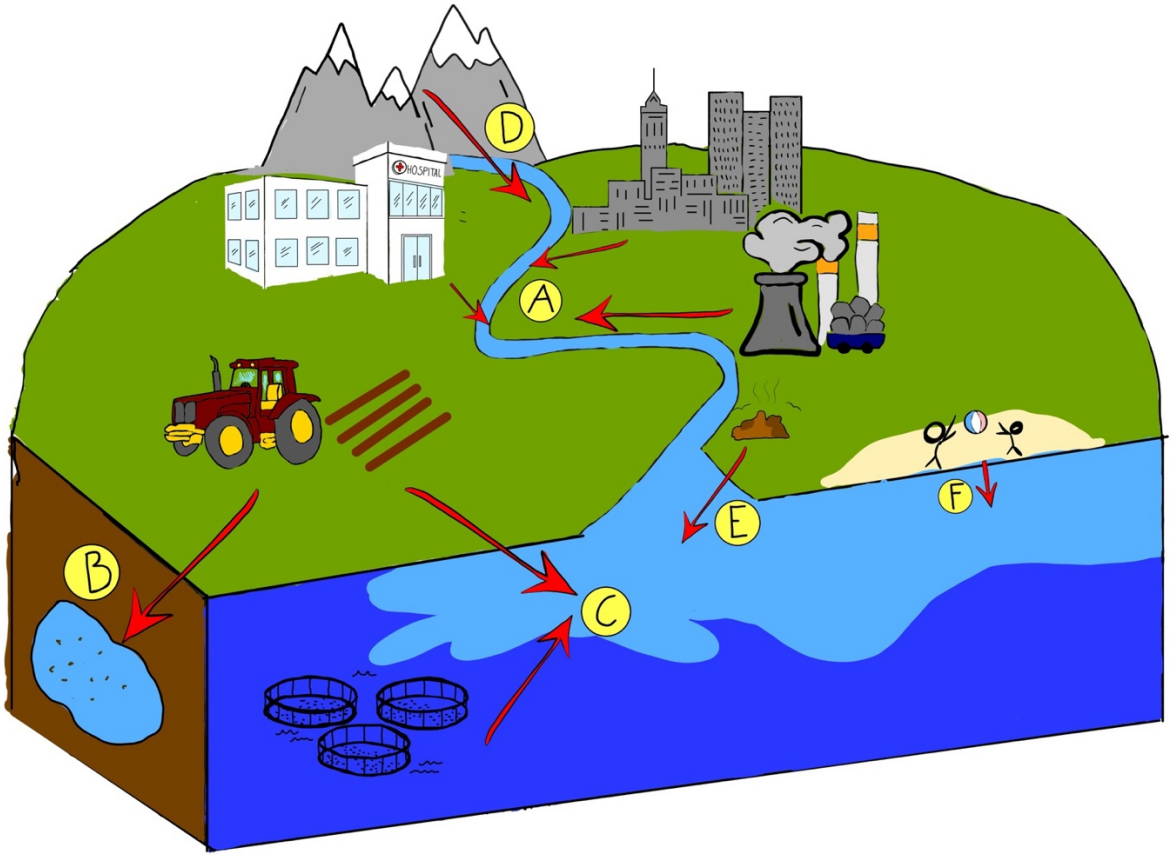

Figure 1: *Potential pathways for MPs, antibiotics, and ARGs/ARB to enter the environment.* ***A:*** *Domestic and clinical waste.* ***B:*** *Groundwater,* ***C:*** *Agriculture and aquaculture,* ***D:*** *Ice melting,* ***E:*** *Wastewater and sewage,* ***F:*** *Recreational wastes.*

The hydrophobic nature of plastic surfaces accommodates for the development of biofilms (Marathe and Bank, 2022). Biofilms occur when bacteria attach themselves to surfaces and form a matrix of organic polymers (Crosta *et al.*, 2022). As previously stated, those found on plastic surfaces are distinct from those in the surrounding environment, making up the plastisphere

. Due to the contemporaneity of antibiotics and plastics, the HGT of ARGs is probabilistic. This is important in considering the durability and dispersal ability of plastics and their

residency time in the natural environment. Especially when it comes to the spread of AMR or potentially opportunistic pathogens.

Aim

This study aims to investigate if polymer type and their surrounding environment, impact biofilm composition and presence of AMR on plastic particles.

Objectives

1) To perform controlled lab experiments using Polyethylene (PE), polystyrene (PS), and polyamide (PA), seawater (SW) or WW for biofilm formation.
2) Isolation and identification of bacteria from the biofilms and test for antibiotic susceptibility
3) Whole genome sequencing and sequence analysis.

Materials and methods

Seawater Collection

Seawater (SW) was collected on the 17th of April 2024 just outside the Institute of Marine Research (IMR) in Bergen, Norway (60.400613°N, 5.306023°E), using a 500 mL flask that had been sterilized with ethanol and air-dried under an extraction hood and a rope. Immediately after collection, the salinity and temperature of the SW were measured using a probe.

At the time of collection, the SW was 7ºC and 13.6ppt in salinity.

Experiment

Polyethylene (PE), polystyrene (PS), and polyamide (PA) beads were washed with 99.9% ethanol for the purpose of sterilization and then air-dried under an extraction hood. Using sterilized metal tweezers, 12 PE and PS beads were added to 6 sterile 11 mL glass test tubes each for each polymer type (Figure 3). For PA, 18 beads were added to 6 test tubes each. The difference in the number of beads was determined to ensure that each tube had approximately 3 cm^3 of plastic surface area. Using a pipette, 5 mL of WW influent, collected from Holen in February 2023, was added to three test tubes containing PA, PS, and PE beads. The same procedure was repeated for the SW samples.

Two tubes, one containing WW and the other containing SW, were set aside without any polymer beads for control purposes.

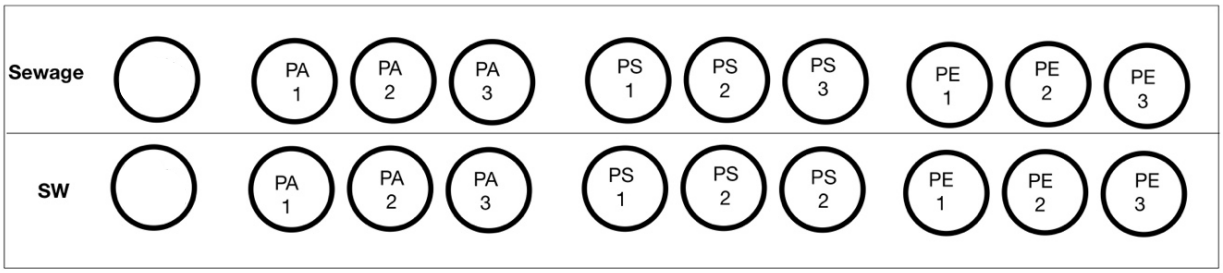

Figure 2: *Schematic representation of the experimental test tube setup. The experiment involved three sets of each polymer type—polyethylene (PE), polystyrene (PS), and polyamide (PA)—with duplicates for both sewage and seawater media.*

Each tube was assigned a number to simplify further analysis (Table 1).

Table 1: *Assigned number for each sample.*

Sample	**Number**
Background WW	1
Background SW	2
Sewage PA 1	3
Sewage PA 2	4
Sewage PA 3	5
SW PA 1	6
SW PA 2	7
SW PA 3	8
Sewage PS 1	9
Sewage PS 2	10
Sewage PS 3	11
SW PS 1	12
SW PS 2	13
SW PS 3	14
Sewage PE 1	15
Sewage PE 2	16
Sewage PE 3	17
SW PE 1	18
SW PE 2	19
SW PE 3	20

All test tubes were placed in an orbitally shaking incubator set at 25°C and 150 RPM for five weeks.

After five weeks of shaking incubation, the polymer beads were washed with sterile water to remove excess bacteria and transferred to 50 mL Falcon tubes. These tubes were then filled with 5 mL of Phosphate Buffered Saline (PBS) and approximately 5 glass beads, followed by being vortexed at maximum speed for 90 seconds to dislodge biofilms and create suspensions. The suspensions were serially diluted by tenfold and plated for the isolation of bacteria.

Isolation of bacteria

Marine agar (MA) with and without 100 µg/mL ampicillin (AMP) and MacConkey agar (MAC) and Chromagar (ECC) with and without 4 µg/mL cefotaxime (FOT) were prepared and poured into Petri dishes. The amount of antibiotics was determined following the EUCAST breakpoint table v.14.0 (EUCAST, 2024). Serial dilutions of SW and WW influent prior to the five-week incubation period were arranged and then plated on MA with and without AMP, and MAC with and without FOT and incubated for 24-48 hours. Plates on MA were incubated at 25°C, while those on MAC and ECC were incubated at 37°C.

After incubation, colonies were counted, and up to five colonies from each growth medium were selected based on morphological diversity. Identification of colonies took place at IMR using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany). Isolates were then stored by transferring them into 700 µL Mueller Hinton Broth (MHB) in 1.5 mL Eppendorf tubes using 10 µL loops. After incubation at either 37°C or 25°C, depending on whether the isolate was derived from MAC, or MA, 200 µL of glycerol was added to the tubes for storage.

Antibiotic Susceptibility Testing (AST)

Isolates were removed from storage by being placed in a Styrofoam box filled with crushed ice to assure that none of the bacteria died. They were restreaked onto MHA and incubated in either 25°C or 37°C depending on what medium they originated, for 24-48h. Tubes of 2 mL of sterile saline were prepared and using a 10 µL loop, bacterial colonies were transferred and suspended into the tubes to obtain 0.5 McFarland turbidity standard. From the suspension, 30 µLwas transferred into Sensititre MHB (Thermo ScientificTM, USA). AST was performed using Sensititre® EUVSEC3 plates (Thermo ScientificTM, USA), according to the protocol of the manufacturers. Minimum inhibitory concentrations (MICs) were determined for ampicillin (AMP) (1–32 µg/mL), meropenem (MERO) (0.03–16 µg/mL), ciprofloxacin (CIP) (0.015-8 µg/mL), azithromycin (AZI) (2-64 µg/mL), amikacin (AMI) (4-128 µg/mL), gentamicin (GEN) (0.5–16 µg/mL), tigecycline (TGC) (0.25–8 µg/mL), ceftazidime (TAZ) (0.25–8 µg/mL), cefotaxime (FOT) (0.25-4 µg/mL), chloramphenicol (CHL) (8-64 µg/mL), colistin (COL) (1- 16 µg/mL), nalidixic acid (NAL) (4-64 µg/mL), tetracycline (TET) (2-32 µg/mL), trimethoprim (TMP) (0.25-16 µg/mL), and sulfamethoxazole (SMX) (8-512 µg/mL). E. coli strain CCUG 17620 was used as negative control. Due to insufficient quantity of equipment, some AST was performed using Sensititre® EUVSEC plates (Thermo ScientificTM, USA) were the minimum MICs were determined for SMX (8-1024 µg/mL), TMP (0.25-32 µg/mL), CIP (0.015-8 µg/mL), TET (2-64 µg/mL), MERO (0.03-16 µg/mL), AZI (2-64 µg/mL), NAL (4-128 µg/mL), CHL (8-128 µg/mL), FOT (0.25-4 µg/mL). TGC (0.25-8 µg/mL), TAZ (0.5-8 µg/mL), AMP (1-64 µg/mL) and GEN (0.5-32 µg/mL).

Molecular methods

DNA extraction

DNA was extracted from selected isolated that were multidrug resistant. DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Germany) following manual instructions described in the following paragraphs.

Under an extraction hood, 220 µL of PBS was added to 1.5 mL Eppendorf tubes. Using a 1µL, suspensions from selected isolates grown on MHA were made. 20 µl proteinase K was then

pipetted into the tubes to ensure the breakdown of proteins that may interfere with DNA extraction. Then 200 µL of the kits buffer AL was added to the samples and incubated at 56° C to optimize proteinase K activity for 10 min so that lysis could occur so that DNA was released into the solution. Thereafter the mixture is transferred to the kits spin and collection tube and centrifuged at 12 000 rpm for 1 min. The collection tube was discarded and new collection tube in addition to a washing buffer was added and centrifuged again to rinse away impurities. Then the collection tube is once again discarded and replaced for a new washer buffer is added prior to centrifuging at 14 000 rpm for 3 min to wash away any remaining contaminants. Both these buffers have had ethanol added to them prior to use and acts to purify the DNA. Then the spin tube was transferred to a new 1.5 mL Eppendorf tube, adding an elution buffer and centrifuged for 1 min at 12 000 rpm to collect the DNA downstream to the Eppendorf tube.

Fluorometry

DNA concentration was then calculated using the Qubit™ 2.0 Fluorometer.

Whole Genome Sequencing (WGS)

21 isolates were sent for whole genome sequencing (WGS) carried out by Eurofins Genomics using the same methodologies described by Radisic *et al.*, (2024).

Statistical analysis

Data provided by MALDI TOF MS identifications were combined and assorted to matrix and polymer type in Excel (Microsoft Corporation, 2024). A three-way analysis of variance (ANOVA) was performed in R studios (RStudio Team, 2022) to understand the interaction of matrix, polymer type and species.

Results

Background bacterial diversity

WW obtained more colonial growth compared to SW. From 25 selected colonies, 6 were not able to be identified by MALDI-TOF MS. From WW, the majority of colonies grew on MA. Colonies from SW exclusively grew on MA.

From the background samples, MALDI-TOF MS only detected two genus's of bacteria each for WW and SW. Those were *Pseudomonas spp*. and *Psychobacter spp*. from WW, and *Buttiauxella spp*. and *Exiguabacterium sp*. from SW (figure 3). From *Pseudomonas* spp., *P. antarctica, extremientialis, ludensis, tolaasii, chlororaphis*, and *koreensis* were all present. Among the *Psychobacter* species, *P. immobilis* was the most abundant (n=4), followed by *P. pulmonis* (n=3), and *P. maritinus* (n=1).

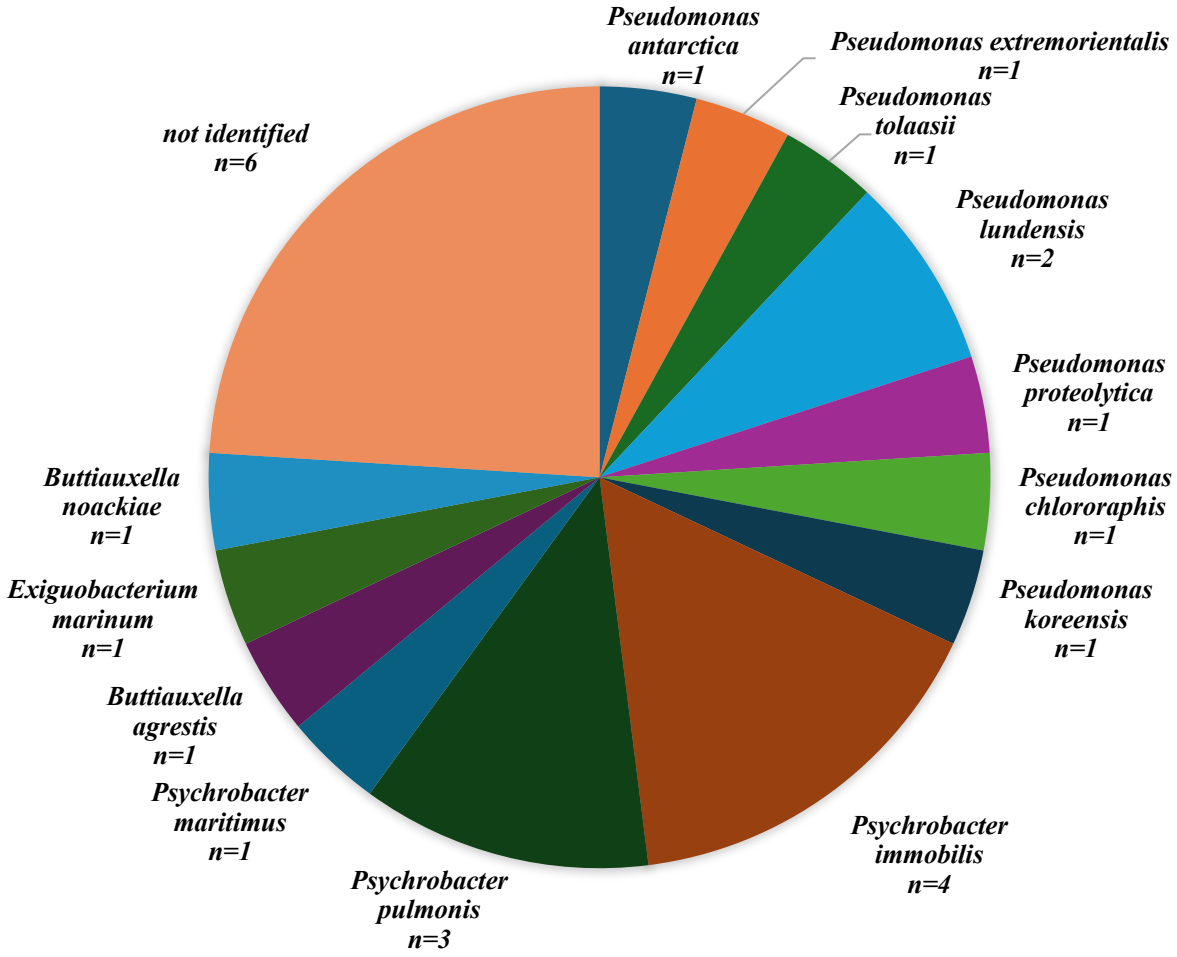

Figure 3: *Pie chart illustrating the proportions and counts of identified bacterial species found in background samples of sewage and seawater. Each colour corresponds to a distinct bacterial species.*

Isolation and identification of bacteria from the controlled experiment

After 5 weeks of incubation, suspensions of samples were streaked, incubated and colony forming units (CFU) was counted. Most samples favoured growth on marine agar (MA) compared to all other mediums (figure 4). However, MacConkey agar (MAC) generally showed the most amount of growth among all samples. Maximum five colonies were selected from each sample and medium for identification and isolation.

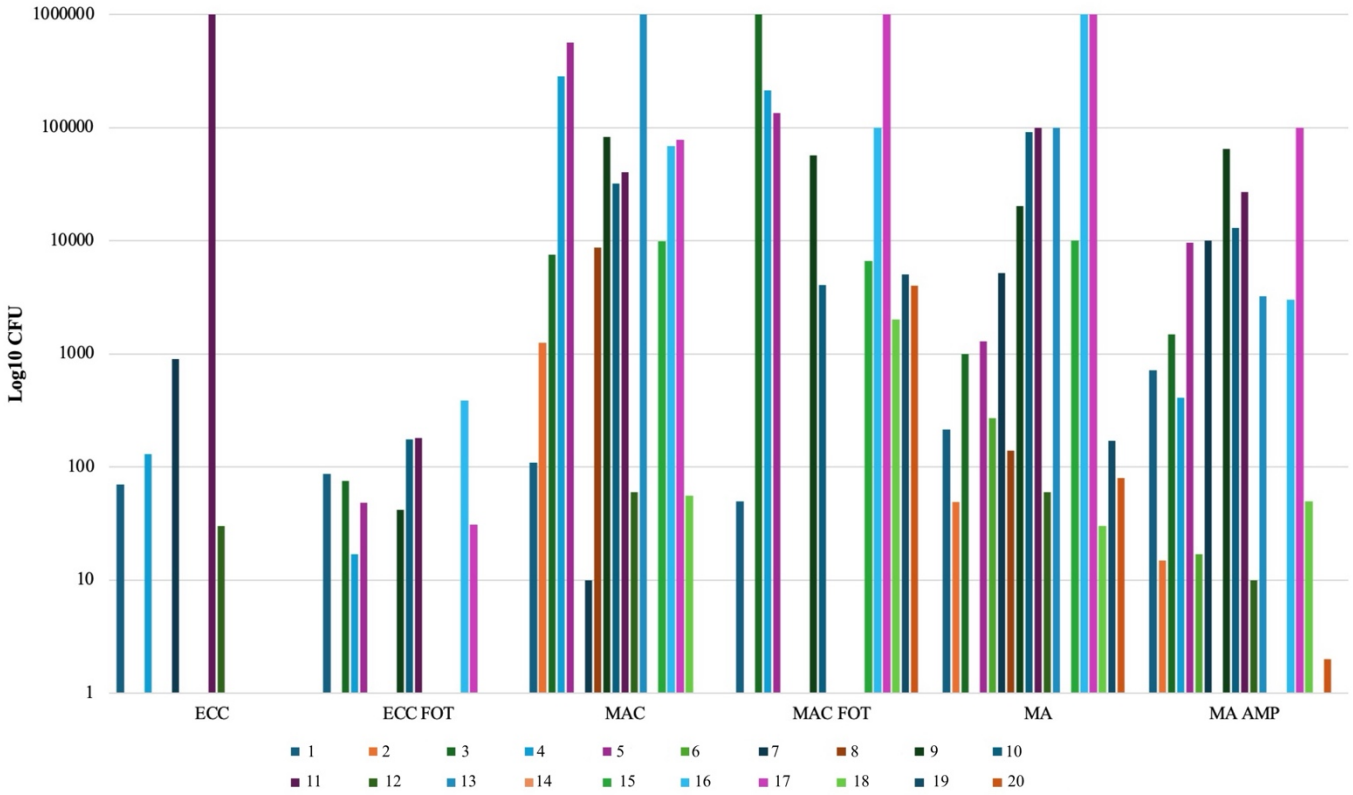

Figure 4: *CFU shown in Log10 from different agars with or without antibiotics. That is Marine agar (MA) with and without 100 µg/mL ampicillin (AMP) and MacConkey agar (MAC) and E. Coli Chromagar (ECC) with and without 4 µg/mL cefotaxime (FOT). The different colours represent different samples indicated by the key below.*

WW had a higher number of colonies compared to SW. WW and SW had common species that grew on them. The majority of the colonies selected from the plates were identified as Gram-negative bacterium by MALDI-TOF MS. Most colonies were identified as *Pseudomonas spp*., followed by bacteria that were not identified, then *Klebsiella sp*. (figure 5). Of *Pseudomonas* spp., *P. rhodesiae* was the most abundant (n=37), followed by *P. koreensis* (n=26), and *P. Protegens* (n=14).

Opportunistic pathogen *K. oxytoca* was isolated from all polymer types and in all matrixes. However, it was especially abundant on PA incubated in SW.

Abundance of present bacteria in samples after 5 week incubation period

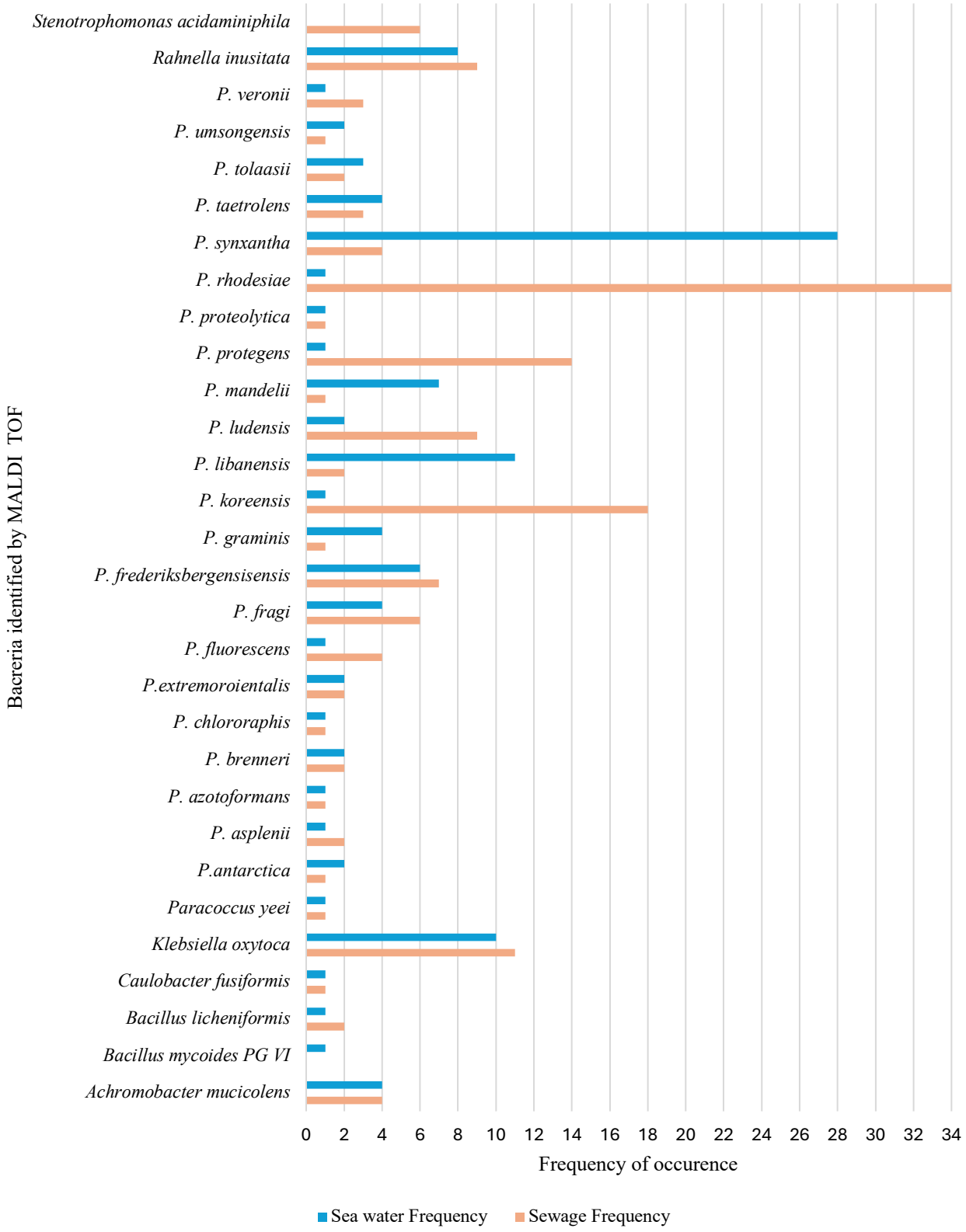

Figure 5: *Bar chart showing the abundance and diversity of bacterial species identified by MALDI-TOF MS from PA, PS, and PE beads incubated at 20°C for a 5-week period. The frequency of occurrence for each species is indicated from sewage and seawater samples with different colours.*

Of the bacteria identified by MALDI-TOF MS from the colonies randomly selected, PA was the most abundant in *P. protegens*, PS was the most abundant in *P. rhodesiae*, and polyethylene (PE) was the most abundant in *P. koreensis* (figure 6). PS demonstrated the greatest diversity of species, followed by PE then PA.

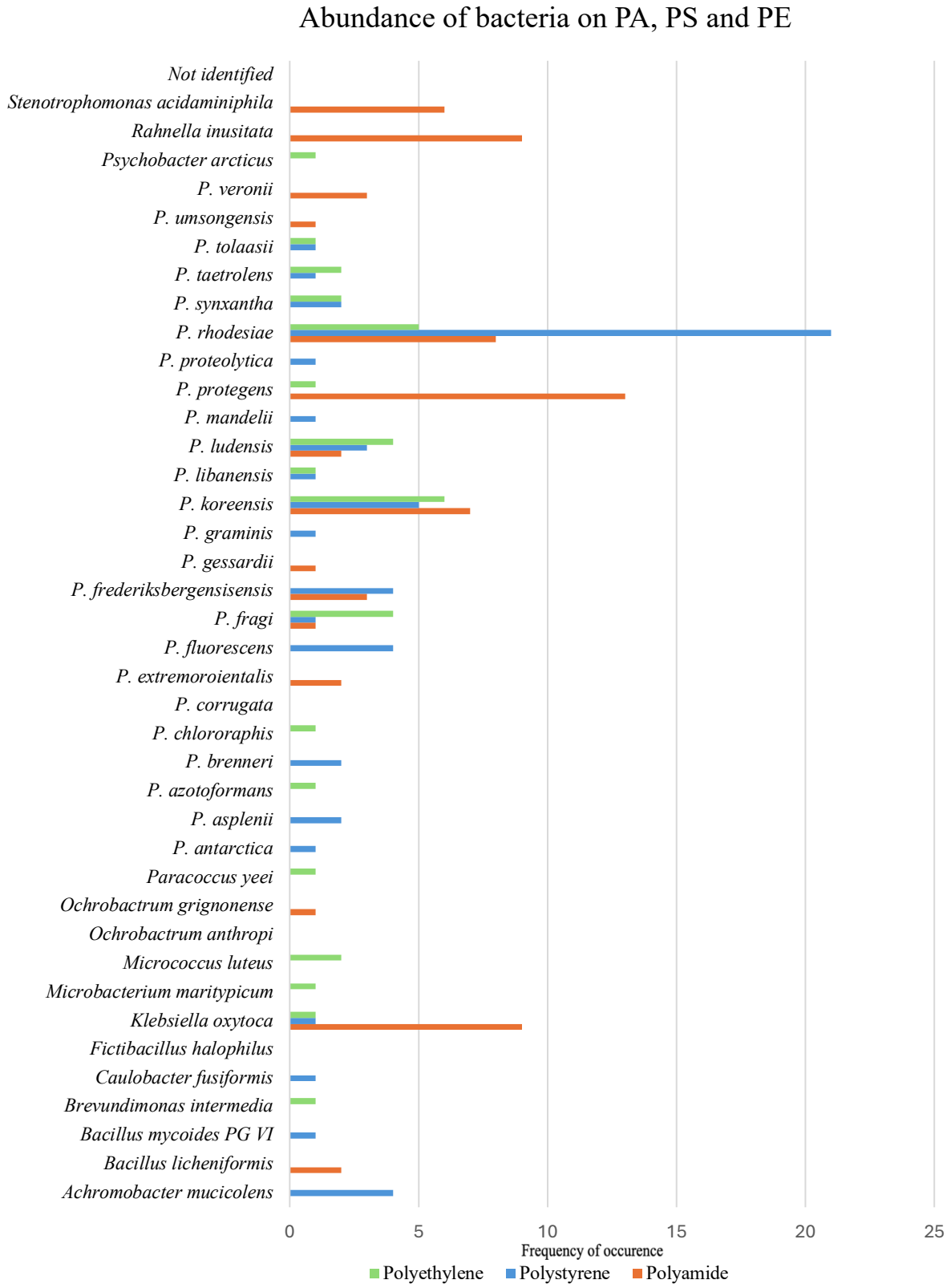

Figure 6: Bar chart showing the abundance and diversity of bacterial species identified by MALDI-TOF MS from PA, PS, and PE beads incubated at 20°C for a 5-week period. The frequency of occurrence for each species is indicated from PE, PS, and PA samples with different colours.

The three-way ANOVA test revealed no statistically significant effect of factors Matrix (P value=0.648), Polymer (P value= 0.388) or Species (P value=0.198) or the following interactions; matrix and polymer (P value=0.565), matrix and species (P value=0253), and species and polymer (P value =0.183) (table 2).

Table 2: *Three-way ANOVA results*

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Matrix	1	1.5	1.541	0.236	0.648
Polymer	2	15.0	7.518	1.151	0.388
Species	29	411.5	14.191	2.172	0.198
Matrix:Polymer	1	2.5	2.480	0.380	0.565
Matrix:Species	10	122.5	12.246	1.874	0.253
Polymer:Species	5	77	15.405	2.358	0.184
Residuals	5	32.7	6.533		

Degrees of freedom (Df), Sum of squares (Sum sq), Mean square (Mean Sq), F value, P value (Pr(>F)).

AST was performed on 72 Gram-negative isolates mainly using EUVSEC3 plates. EUVSEC plates was used on 27 samples due to insufficient materials. Isolates tested with EUVSEC3 were tested against 15 antibiotics whereas EUVSEC tested against 14 antibiotics to determine their minimum inhibitory concentration (MIC) (Table3).

All isolates were resistant to AMP except for 1 MAC 4, 9 MAC FOT 3, 12 MAC 1 and 13 MAC 2. 4 MAC FOT 1, 12 MAC 3, and 3 ECC 1 showed only some signs of resistance to AMP. *Pseudomonas spp*. demonstrated a wide range in terms of resistance.
Furthermore, all isolates demonstrated high resistance to FOT with the exception of WW samples grown on PA (3 ECC 1,2 and 4), and SW samples from PA (7 MA AMP 2, and 8 MAC 3, 4 and 5) and PS (12 MAC 1 and 3, and 13 MAC 2).

Table 3: *Minimum inhibitory concentrations (MIC) for 72 isolates against 15 antibiotics. The concentrations have been marked as low or high MIC as clinical breakpoints are not available for all the species studied here. Antimicrobic resistance (AMR) is indicated by low (green) or high (red).*

Polymer	Matrix	Isolate	MALDI-TOF MS	MIC (µG/100 mL)														
				AMP	MERO	CIP	AZI	AMI	GEN	TGC	TAZ	FOT	CHL	COL	NAL	TET	TMP	SMX
	WW	1 ECC FOT 5	*Pseudomonas koreensis*	>32	1	0.12	64	<4	<0.5	1	>8	>4	>64	4	64	8	>16	256
	WW	1 MAC 4	*No ID*	<1	0.12	0.25	64	<4	<0.5	<0.25	4	0.5	<8	>16	32	<2	<0.25	64
PA	WW	3 ECC 1	*NO ID*	8	16	9		>128	<0.5	>8	0.5	0.5	64	<1	>64	<2	>16	<8
PA	WW	3 ECC 2	*No ID*	32	16	9	64	>128	<0.5	>8	<0.25	0.5	64	2	>64	<2	>16	<8
PA	WW	3 ECC 4	*No ID*	>32	1	0.06	32	<4	1	1	2	4	>64	2	8	4	>16	64
PA	WW	3 MA 2	*Pseudomonas frederiksberg*	>32	0.5	0.12	>64	<4	1	2	>8	>4	>64	<1	16	2	>16	>512
PA	WW	3 MA 4	*Ochrobactrum grignonense*	>32	2	0.25	16	32	>16	1	>8	>4	32	>16	<4	<2	>16	>512
PA	WW	*3 MA AMP 1*	*Pseudomonas extremiorientalis*	>32	8	0.5	64	<4	1	2	>8	>4	>64	>16	64	8	>16	>512
PA	WW	3 MA AMP 2	*Pseudomonas frederiksberg*	>32	0.12	0.12	>64	<4	<0.5	1	>8	>4	>64	<1	16	4	>16	>512
PA	WW	3 MA AMP 4	*Pseudomonas frederiksberg*	>32	0.12	0.12	> 64	<4	1	2	>8	>4	>64	<1	16	4	>16	>512
PA	WW	3 MAC 5	No ID	>32	1	0.06	32	<4	<0.5	<0.25	1	>4	16	<1	16	<2	>16	<8
PA	WW	4 MA 1	*Pseudomonas frederiksberg*	>32	0.12	0.25	>64	8	8	4	8	>4	>64	<1	<4	<2	>16	>512
PA	WW	4 MA 2	*Pseudomonas veronii*	>32	16	0.25	>64	<4	<0.5	0.5	4	>4	32	<1	8	4	>16	512
PA	WW	4 MAC 4.1	*Pseudomonas graminis*	>32	4	0.12	>64	<4	<0.5	2	8	>4	>64	<1	32	4	>16	>512
PA	WW	4 MAC 4.2	*Pseudomonas koreensis*	>32	0.5	0.12	64	<4	4	4	>8	>4	32	<1	64	4	>16	>512

PA	WW	4 MAC 5	Pseudomonas koreensis	>32	0.12	0.06	4	<4	<0.5	<0.25	>8	>4	32	<1	32	<2	>16	<8
PA	WW	4 MAC FOT 1	*Achromobacter piechaudii*	8	0.06	0.5	16	<4	<0.5	<0.25	1	>4	8	<1	8	<2	>16	<8
PA	WW	5 ECC FOT 1	*Pseudomonas protegens*	>64	>16	0.5	>64	NA	4	8	>8	>4	64	>16	64	8	>32	>1024
PA	WW	5 ECC FOT 4	*Pseudomonas protegens*	>64	>16	0.5	>64	NA	4	8	>8	>4	>128	>16	32	8	>32	128
PA	WW	5 MA 1	*Klebsiella oxytoca*	>32	>16	0.12	>64	<4	1	4	2	>4	>64	>16	16	8	>16	>512
PA	WW	5 MA 2	*No identification*	>32	1	0.06	32	<4	<0.5	1	1	>4	16	8	32	<2	>16	128
PA	WW	5 MA 3	*Pseudomonas protegens*	>64	8	1	>64	NA	2	8	>8	>4	>128	>16	32	8	>32	1024
PA	WW	5 MA 4	*Pseudomonas ludensis*	>64	16	0.25	>64	NA	8	8	>8	>4	>128	>16	64	16	>32	>1024
PA	WW	5 MA 5	*Pseudomonas protegens*	>64	4	0.12	>64	NA	2	2	>8	>4	128	2	64	4	>32	1024
PA	WW	5 MA AMP 1	*Pseudomonas ludensis*	>32	2	0.06	>64	<4	<0.5	2	4	>4	64	<1	16	4	>16	64
PA	WW	5 MA AMP 2	*Pseudomonas rhodesiae*	>32	2	0.12	>64	<4	2	2	4	>4	>64	4	16	4	>16	>512
PA	WW	5 MA AMP 3	*Pseudomonas veronii*	>32	0.12	0.12	>64	<4	<0.5	2	4	>4	64	<1	16	4	>16	64
PA	WW	5 MAC 3	*Pseudomonas protegens*	>64	8	1	>64	NA	4	8	>8	>4	>128	16	32	16	>32	1024
PA	WW	5 MAC 5	*Pseudomonas protegens*	>64	>16	0.5	>64	NA	2	8	8	>4	>128	>16	32	16	>32	512
PA	WW	5 MAC FOT 3	*Pseudomonas protegens*	>64	>16	1	>64	NA	8	8	8	>4	128	16	64	8	>32	256
PA	SW	6 MAC 2 (1)	*Pseudomonas ludensis*	>32	0.5	0.06	8	<4	<0.5	0.5	4	>4	32	<1	8	4	>16	512

PA	SW	6 MAC FOT 2	*Pseudomonas extremiorientalis*	>32	16	1	2	<4	<0.5	1	>8	>4	<8	16	<4	8	>16	>512
PA	SW	7 MA 3	*Rahnella inusitata*	>64	0.5	0.12	>64	NA	1	2	8	>4	128	4	32	4	>32	512
PA	SW	7 MA 5	*Rahnella inusitata*	>64	4	0.25	>64	NA	1	2	8	>4	128	2	16	16	>32	>1024
PA	SW	7 MA AMP 2	*Pseudomonas rhodesiae*	>64	4	0.25	64	NA	>32	2	<0.5	<0.25	16	8	>128	8	>32	>1024
PA	SW	7 MA AMP 4	*Rahnella inusitata*	>64	16	0.12	>64	NA	2	2	>8	>4	128	<1	16	16	>32	1024
PA	SW	7 MA AMP 5	*Pseudomonas rhodesiae*	>32	2	0.12	>64	<4	2	2	4	>4	>64	2	8	16	>16	>512
PA	SW	8 MAC 3	*Klebsiella oxytoca*	>32	0.06	1	<2	<4	<0.5	<0.25	2	<0.25	<8	<1	<4	<2	16	<8
PA	SW	8 MAC 4	*Klebsiella oxytoca*	>32	0.12	1	>64	<4	<0.5	<0.25	2	<0.25	<8	<1	<4	<2	>16	<8
PA	SW	8 MAC 5	*Klebsiella oxytoca*	>32	0.12	1	<2	<4	<0.5	<0.25	2	<0.25	64	<1	<4	<2	>16	<8
PA	SW	8 MAC FOT 5	*Klebsiella oxytoca*	>32	>16	>9	16	32	<0.5	<0.25	>8	4	16	<1	<4	>32	<0.25	<8
PS	WW	9 MA 4	*Pseudomonas rhodesiae*	>64	4	8	>64	NA	2	2	>8	>4	128	16	128	4	>32	256
PS	WW	9 MA AMP 1	*Pseudomonas taetrolens*	>64	4	0.25	>64	NA	1	2	8	>4	128	2	64	4	>32	>1024
PS	WW	9 MA AMP 2	*Pseudomonas rhodesiae*	>64	8	0.25	>64	NA	2	2	>8	>4	128	>16	32	4	>32	256
PS	WW	9 MAC 4 (1)	Klebsiella oxytoca	>32	8	9	>64	>128	<0.5	>8	<0.25	4	>64	<1	>64	<2	>16	128
PS	WW	9 MAC FOT 3	*Pseudomonas rhodesiae*	<1	0.25	0.25	<2	<4	<0.5	<0.26	8	2	<8	>16	64	<2	16	>512
PS	WW	10 ECC FOT 5	*Pseudomonas fluoresens*	>32	2	1	16	<4	<0.5	0.5	>8	>4	32	<1	16	8	>16	>512

PS	WW	10 MA 1	*Pseudomonas rhodesiae*	>64	4	0.12	>64	NA	1	2	8	>4	128	4	16	4	>32	256
PS	WW	10 MA 2	*Pseudomonas rhodesiae*	>64	4	4	>64	NA	2	2	>8	>4	128	16	64	16	>32	1024
PS	WW	10 MA 3	*Pseudomonas rhodesiae*	>64	4	4	>64	NA	1	2	>8	>4	128	16	128	4	>32	1024
PS	WW	10 MA 4	*Pseudomonas rhodesiae*	>32	2	0.12	>64	<4	1	2	>8	>4	>64	8	32	4	>16	>512
PS	WW	10 MA 5	*Pseudomonas rhodesiae*	>64	4	0.25	>64	NA	2	4	8	>4	128	4	32	4	>32	256
PS	WW	11 ECC FOT 1	*Not identified*	>32	2	0.06	16	<4	<0.5	0.5	8	>4	16	<1	64	<2	>16	>512
PS	WW	11 MAC FOT 2	*Pseudomonas ludensis*	>64	>16	0.12	>64	NA	<0.5	4	>8	>4	>128	<1	32	16	>32	>1024
PS	SW	12 MA 1	*Pseudomonas rhodesiae*	>32	2	0.12	>64	<4	1	2	4	>4	>64	<1	8	4	>16	64
PS	SW	12 MA 2	*Pseudomonas fragi*	>64	4	0.25	>64	NA	2	4	4	>4	128	4	16	4	>32	>1024
PS	SW	12 MA AMP 2	*Pseudomonas ludensis*	>32	2	0.5	>64	<4	1	1	4	>4	>64	4	16	4	>16	128
PS	SW	12 MAC 1	*Pseudomonas ludensis*	<1	0.12	0.25	<2	<4	<0.5	<0.25	8	1	<8	>16	64	<2	0.5	<8
PS	SW	12 MAC 3	*Pseudomonas ludensis*	16	>16	>9	>64	4	2	<0.25	2	<0.25	>64	4	16	<2	>16	<8
PS	SW	12 MAC 3	*Pseudomonas ludensis*	>64	2	0.12	64	<4	4	4	>8	>4	>128	4	16	16	>32	>1024
PS	SW	13 MAC 1	*Pseudomonas ludensis*	>64	4	0.25	64	NA	<0.5	1	4	>4	128	2	32	4	>32	512
PS	SW	13 MAC 2	*Pseudomonas rhodesiae*	<1	>16	>9	>64	64	4	1	4	1	>64	8	32	16	>16	<8
PS	SW	14 ECC FOT 3	*Not identified*	>32	8	1	<2	8	2	<0.25	8	>4	<8	<1	<4	32	>16	>512

PE	WW	15 MA AMP 1	*Pseudomonas fragi*	>32	2	0.12	>64	<4	2	2	4	>4	64	2	16	4	>16	>512
PE	WW	15 MA AMP 2	*Pseudomonas fragi*	>64	4	0.5	>64	NA	1	2	8	>4	128	16	16	4	32	1024
PE	WW	15 MA AMP 4	*Pseudomonas ludensis*	>64	4	0.25	>64	NA	1	2	8	>4	16	8	128	4	>32	1024
PE	WW	15 MA AMP 5	*Pseudomonas ludensis*	>64	0.5	0.12	>64	NA	<0.5	2	8	>4	128	4	32	4	>32	512
PE	WW	15 MAC 4	*Pseudomonas fluoresens*	>32	>16	1	>64	<4	<0.5	>8	>8	>4	>64	>16	>64	16	>16	512
PE	WW	16 MA 1	*Pseudomonas protegens*	>64	4	2	>64	NA	2	2	>8	>4	128	2	64	4	>32	256
PE	WW	16 MAC 4	*Not identified*	>64	4	0.25	>64	NA	<0.5	4	>8	>4	>128	<1	32	32	>32	>1024
PE	WW	17 ECC FOT 1	*Pseudomonas rhodesiae*	>32	0.12	0.06	32	<4	<0.5	<0.25	>8	>4	64	<1	32	4	>16	>512
PE	WW	17 MAC FOT 1	*Pseudomonas ludensis*	>32	1	0.25	32	<4	<0.5	2	4	>4	64	8	32	8	>16	256
PE	WW	17 MAC FOT 3	*Pseudomonas ludensis*	>32	2	0.12	16	<4	<0.5	<0.25	4	>4	64	<1	16	8	>16	>512

Ampicillin (AMP), meropenem (MERO), ciprofloxacin (CIP), azithromycin (AZI), amikacin (AMI), gentamicin (GEN), tigecycline (TGC), ceftazidime (TAZ), cefotaxime (FOT), chloramphenicol (CHL), colistin (COL), nalidixic acid (NAL), tetracycline (TET), trimethoprim (TMP), and sulfamethoxazole (SMX). Isolates are defined by their assigned number, medium of growth then colony count number.

DNA

DNA was extracted and quantified in ng/µL using Qbit 2.0 Fluorometer. Isolates selected for DNA extractions were based on MERO resistance and representation in terms of polymer type and matrix. The broadscale DNA concentration ranged from no DNA to 149 ng/µL (table 3).

Table 4: *DNA concentrations of 21 samples by Qubit 2.0 fluorometer.*

Isolate	Qubit 2.0 Fluorometer (ng/µL)
3 MA 4	149
3 MA AMP 1	50.2
4 MAC 4.1	15
4 MAC 4.2	36.4
5 ECC FOT 4	61.6
5 MAC 3	11.2
5 MAC 5	19.7
5 MAC FOT 3	45.6
5 MA 1	27
5 MA 2	35.4
5 MA 3	26
7 MA AMP 5	51.6
8 MAC 4	175
9 MAC 4	4.8
9 MA 4	13.6
9 MA AMP 2	14.5
10 MA 4.1	52.8
10 MA 4.2	30.6
10 MA 5	35.6
11 ECC FOT 1	55.2
13 MAC 2	8.82

Discussion

This study presents a controlled laboratory experiment where PA, PE and PS beads were immersed in SW or WW influent, orbitally incubated at 25°C for a duration of five weeks. Then, suspensions were made to cultivate colonies from the biofilms of the plastic beads, some of which were selected based on morphology to be further identified. All selected colonies were re-streaked to form isolates and underwent AST. 21 isolates were appointed for WGS with selection based on meropenem resistance and/or diversity and representation from the entire sample size.

Impact of polymer type and environment on biofilm composition

Statistical analysis demonstrated no significant impact of polymer type, matrix and species. This may be due to the limitation of methodology used. Culture based methods have limited depth when it comes to exploring microbiota. This is attributed to the fact that most of the microbes in the environment are difficult to culture in the lab and represent huge diversity of uncultured bacteria (Kaeberlein *et al.*, 2002; Stewart, 2012). Having said that, we have isolated highly resistant strains from plastic biofilms but not from the background samples. Thus, suggesting that biofilms may enrich antibiotic resistant bacteria. This is consistent with the previous studies showing enrichment of resistant bacteria on plastic surfaces (Laganà *et al.*, 2019; Meng *et al.*, 2021). Even though the matrices were different in this study, the controlled factors, i.e., temperature and orbital motion, remained consistent throughout. As such, the uniform controlled conditions may have played a significant role in similarity of the taxa of isolates obtained across polymer types and matrices as some studies have found seasonality and geographics play an important role in the biofilm composition (Oberbeckmann *et al.*, 2014; Sérvulo *et al.*, 2023).

Microbial community on different plastic polymers

Biofilms from PS were the most biodiverse in our study. Several classes of bacteria have been reported on the biofilms of PS (Tourova *et al.*, 2020). When compared to other polymer types, PS was found to be favour pathogens in biofilm (Yang *et al.*, 2022), especially *Klebsiella*, which is consistent with the findings of this study. Biofilms on PA had the least amount of biodiversity. In this experiment, the beads were smaller, hence the relative surface area was increased compared to those of PE and PS. In PE, this phenomenon has been proven to give rise to more distinct microbial communities (Zhu *et al.*, 2022), and could have the same effect for PA though further research is needed.

Bacteria present on marine plastic analysed using culturing-based methods

Pseudomonas spp.

Pseudomonas was the most abundant genus among all matrices and polymer types. This is a widespread genus found in all natural environments i.e., terrestrial, fresh water and marine, in addition to plants and animals (Spiers *et al.*, 2000), and also the plastisphere (Park *et al.*, 2023). In fact, their presence in plastic biofilm is common in the marine environment (Zhai *et al.*, 2023).

Some *Pseudomonas sp*. are lipase-positive (Yang *et al.*, 2022; Lv *et al.*, 2024), meaning they carry an enzyme which can break triglycerides and glycerol down (Pirahanchi and Sharma, 2024), and are associated with biofilm formation on plastics (Lv *et al.*, 2024). Furthermore, one study found that the influence of hydrodynamic stress strengthens biofilm formation in P. fluorescens (Jara *et al.*, 2021). Jara et al (2021) report higher CFU count and bacterial density when incubated in an orbitally shaking environment, likely due to higher dispersal of nutrients and oxygen. *Pseudomonas sp*. was found to dominate the biofilms, may be due to characteristics pertaining to the strict aerobic nature of the bacterium itself (Stanier *et al.*, 1966), as well as the nutrients in the immediate environment.

Klebsiella spp.

Opportunistic human pathogen *Klebsiella oxytoca* was isolated from WW samples of PS and PE, and from several SW samples of PA. *K. oxytoca* is commonly found in aquatic and soil environments (Ni *et al.*, 2021) and is commensally present in parts of the human body (Neog *et al.*, 2021). Although typically harmless, it has been reported to cause urinary tract, respiratory, bloodstream, surgical site infections, endocarditis and colitis (Neog *et al.*, 2021; Yang *et al.*, 2022). Some strains exhibit multidrug resistance and can be fatal (Neog *et al.*, 2021).

Antibiotic susceptibility testing (AST) revealed that isolates from SW submerged PA exhibited less resistance across 15 antibiotics. However, isolates from WW samples of PS, such as 9 MAC 4.1, showed significantly higher resistance, with isolate 5 MA 1, incubated on PA in WW demonstrating even greater resistance, particularly to meropenem (MERO) and colistin (COL). We observed in general higher resistance in strains obtained from plastic incubated in WW, suggesting that matrix/environment plays an important role in determining the microbiota of the biofilms in plastics. This is in accordance with the previous studies (Oberbeckmann *et al.*, 2018; Basili *et al.*, 2020). The composition of the WW environment likely contributes to selection pressure, as WW contains high concentrations of antimicrobial compounds, fostering the survival of antibiotic-resistant bacteria (ARB) (Popa et al., 2021; Radisic et al., 2023). Furthermore, horizontal gene transfer (HGT) could facilitate the spread of resistance, as *Klebsiella* species are known to acquire virulence plasmids from other bacteria (Metcalf et al., 2024). Thus, our study also suggests that plastic particles originating in WW are likely to carry pathogenic bacteria that are resistant to multiple antibiotics, although more follow-up studies are needed to ascertain this fact.

Although *K. oxytoca* can often be treated with commonly used antibiotics such as cephalosporins, carbapenems, amikacin, and quinolones (Yang et al., 2022), this study presents strains that have been identified with resistance to a wide range of antibiotics including ampicillin (AMP), MERO, azithromycin (AZI), cefotaxime (FOT), chloramphenicol (CHL), trimethoprim (TMP), nalidixic (NAL), amikacin (AMI) and sulfamethoxazole (SMX). Instances of resistance to AMP, MERO, AZI, FOT, CHL, TMP, NAL, AMI, and SMX have all been reported (Olaitan *et al.*, 2014; Singh, Cariappa and Kaur, 2016; AL-Khikani *et al.*, 2020; Ahmed Hasan and Mohammed Bakr, 2022; Radisic *et al.*, 2024)

ARGs associated with CHL, TMP and SMX has also been identified (Pérez-Vazquez *et al.*, 2019). Notably none of these resistance patterns have been reported together, nor associated to plastic surfaces, highlighting the pressing need for better plastic waste management.
Additionally, *K. oxytoca's* presence in the natural environment may be concerning for the food chain. From a fish market in China, 41 commonly consumed species carried the bacterium and 14 carried virulence associated genes (Ni *et al.*, 2021). These were found to be multidrug resistant, particularly of SMX and TMP (Ni *et al.*, 2021).

AST of isolates from different plastic polymers

Antibiotic susceptibility revealed resistance to last resort antibiotic MERO in 16 isolates and to COL in 15 isolates. From those isolates only *K. oxytoca* represents pathogenic bacteria, although others could be potentially pathogenic. However, this remains a major concern due to the HGT of ARGs (Marathe and Bank, 2022; Stevenson *et al.*, 2024). HGT may occur in the biofilms which is facilitated by close contact of bacteria in biofilms (Marathe and Bank, 2022). This could occur between a non-opportunistic pathogen and a pathogen which could cause disease (Bello-López *et al.*, 2019), either directly to humans, or through marine species which can transferred upwards through trophic levels (Provencher *et al.*, 2019). This is especially concerning considering the dispersal of plastics in the marine environment (Lartaud *et al.*, 2020) and the scale at which plastics are bioaccumulated in the marine food web through ingestion (Provencher *et al.*, 2019).

To identify antibiotic resistant genes (ARGs), WGS must be executed for which results are still awaited. This would specifically provide information about which genes are possessed by these isolates that make them multidrug resistant. To determine this, further research is required.

Limitations

Although we see some differences in the composition of biofilms on different polymers.
A limitation of this study is that only a small proportion of the biofilm has been identified in our culture-dependent approach in the study. Molecular methods like amplicon sequencing of 16S rRNA gene can provide a better picture of the total microbial diversity (Caporaso *et al.*, 2011) and will help us understand the effect of polymer type on microbial diversity in biofilms. Shotgun metagenomics is another method that will provide a picture of not only microbiota but also genes carried by the microbiota. It can provide insights into the ARGs and mobile genetic elements carried by bacteria in the biofilm, thus, providing a complete understanding of microbiome (Quince *et al.*, 2017; Manoharan *et al.*, 2021). We plan to have follow-up study using shotgun metagenomics on the biofilms collected in this study in future.

Another limitation of the study is that we do not know what ARGs are carried by the bacteria isolated in our study. Whole genome sequence analysis is an important tool for understanding the genetic mechanisms of resistance and genetic map of ARGs (Klemm *et al.*, 2018). In order to identify ARGs, we have extracted genomic DNA from selected isolates and send it for whole genome sequencing (WGS). This will provide insights into the genes carried by isolates that make them multidrug resistant and help understand the genetic basis of observed resistance. It will also help understanding the relatedness of different isolates of the same species that were isolated from different matrix.

Conclusions

This study demonstrates that microbes are capable of forming biofilms on PA, PE and PS that show AMR when incubated in both SW and WW. Biofilms on all polymer types and matrices were dominated by *Pseudomonas sp*., likely due to similar environmental conditions and the ecological diversity of this genus. Several bacterial isolates, including the potential pathogen *Klebsiella oxytoca*, were found to be multidrug-resistant when determining the MIC during AST. This study provides further evidence that plastisphere may enrich potential pathogens and AMR. As plastic waste continues to accumulate, particularly in marine environments, this poses an increasing global health risk. Our study shows that pathogenic and multidrug-resistant bacteria can colonize plastics and thus, plastics may spread these entities in the aquatic environment. The plastic pollution not only threatens marine ecosystems but also presents a risk to human and animal health.

Future perspective

We plan to incorporate WGS of isolates and metagenomics of the total DNA from the biofilms. This would offer deeper insights into ARGs present in microbial communities on the surfaces of plastics. It may reveal differences among different polymer types and/or matrices. This will help us understand how AMR potentially spreads in the plastisphere.

Future studies may also investigate if different polymers have effect on microbes in biofilms using field conditions rather that controlled lab experiments as performed here. They can also investigate why PS was the most biodiverse and attracted the highest number of potential pathogens.

Finally, this study highlights the urgent need to develop strategies mitigating plastic pollution which impacts the marine environment the most. Future work could also look into the ecological role of the bacteria present on the biofilm and associated bioaugmentation. Furthermore, efforts should be made into optimizing filtration of WWTP to reduce MPs and antibiotics that reach the marine environments. Stakeholders should be informed to help mitigate the issue of plastic pollution.

References

Ahmed Hasan, S. and Mohammed Bakr, M. (2022) 'Bacteriological and Molecular Detection of Klebsiella oxytoca and its Resistance to Antibiotics among Clinical Specimens from Kirkuk, Iraq', *Archives of Razi Institute*, 77(5), pp. 1521–1525. Available at: https://doi.org/10.22092/ari.2022.357753.2095.

Alduina, R. (2020) 'Antibiotics and Environment', *Antibiotics*, 9(4), p. 202. Available at: https://doi.org/10.3390/antibiotics9040202.

AL-Khikani, F.H.O., Abadi, R.M. and Ayit, A.S. (2020) 'Emerging Carbapenemase Klebsiella oxytoca with Multidrug Resistance Implicated in Urinary Tract Infection', *Biomedical and Biotechnology Research Journal (BBRJ)*, 4(2), p. 148. Available at: https://doi.org/10.4103/bbrj.bbrj_165_19.

Aminov, R.I. and Mackie, R.I. (2007) 'Evolution and ecology of antibiotic resistance genes', *FEMS Microbiology Letters*, 271(2), pp. 147–161. Available at: https://doi.org/10.1111/j.1574-6968.2007.00757.x.

Atanasova, N. S, Stoitsova. T, Puanova-Krasteva. M, Kambourova. (2021) 'Plastic Degradation by Extremophilic Bacteria', *International Journal of Molecular Sciences*, 22(11), p. 5610. Available at: https://doi.org/10.3390/ijms22115610.

Basili, M., Quero, G. M., Giovannelli, D., Manini, E., Vignaroli, C., Avio, C. G., De Marco, R. and Luna, G. M. (2020) 'Major Role of Surrounding Environment in Shaping Biofilm Community Composition on Marine Plastic Debris', *Frontiers in Marine Science*, 7. Available at: https://doi.org/10.3389/fmars.2020.00262.

Beard, D., Clason, C. C., Rangecroft, S., and Poniecka, E. (2022a) 'Anthropogenic contaminants in glacial environments I: Inputs and accumulation', *Progress in Physical Geography: Earth and Environment*, 46(4), pp. 630–648. Available at: https://doi.org/10.1177/03091333221107376.

Bello-López, J. M., Cabrero-Martínez, O. A., Ibáñez-Cervantes, G., Hernández-Cortez, C., Pelcastre-Rodríguez, L. I., Gonzalez-Avila, L. U., and Castro-Escarpulli, G. (2019) 'Horizontal Gene Transfer and Its Association with Antibiotic Resistance in the Genus

Aeromonas spp.', *Microorganisms*, 7(9), p. 363. Available at: https://doi.org/10.3390/microorganisms7090363.

Borrelle, S. B., Ringma, J., Law, K. L., Monnahan, C. C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G. H., Hilleary, M. A., Eriksen, M., Possingham, H. P., De Frond, H., Gerber, L. R., Polidoro, B., Tahir, A., Bernard, M., Mallos, N., Barnes, M. (2020) 'Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution', *Science*, 369(6510), pp. 1515–1518. Available at: https://doi.org/10.1126/science.aba3656.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., and Knight, R. (2011) 'Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample', *Proceedings of the National Academy of Sciences*, 108(supplement_1), pp. 4516–4522. Available at: https://doi.org/10.1073/pnas.1000080107.

Caruso, C., Valluzzi, R. L., Colantuono, S., Gaeta, F., and Romano, A. (2021) 'β-Lactam Allergy and Cross-Reactivity: A Clinician's Guide to Selecting an Alternative Antibiotic', *Journal of Asthma and Allergy*, 14, pp. 31–46. Available at: https://doi.org/10.2147/JAA.S242061.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., and Hanage, W. P. (2015) 'Antibiotics in agriculture and the risk to human health: how worried should we be?', *Evolutionary Applications*, 8(3), pp. 240–247. Available at: https://doi.org/10.1111/eva.12185.

Cholewińska, P., Moniuszko, H., Wojnarowski, K., Pokorny, P., Szeligowska, N., Dobicki, W., Polechoński, R., and Górniak, W. (2022) 'The Occurrence of Microplastics and the Formation of Biofilms by Pathogenic and Opportunistic Bacteria as Threats in Aquaculture', *International Journal of Environmental Research and Public Health*, 19(13), p. 8137. Available at: https://doi.org/10.3390/ijerph19138137.

Chubarenko, I., Bocherikova, I., Esiukova, E., and Isachenko, I. (2023) 'Microplastics in sea ice: A fingerprint of bubble flotation', *Science of The Total Environment*, 892, p. 164611. Available at: https://doi.org/10.1016/j.scitotenv.2023.164611.

Cook, M.A. and Wright, G.D. (2022) ‘The past, present, and future of antibiotics’, *Science Translational Medicine*, 14(657), p. eabo7793. Available at: https://doi.org/10.1126/scitranslmed.abo7793.

Crosta, A., De Felice, B., Antonioli, D., Chiarcos, R., Perin, E., Ortenzi, M. A., Gazzotti, S., Azzoni, R. S., Fugazza, D., Gianotti, V., Laus, M., Diolaiuti, G., Pittino, F., Franzetti, A., Ambrosini, R., and Parolini, M. (2022) ‘Microplastic contamination of supraglacial debris differs among glaciers with different anthropic pressures’, *Science of The Total Environment*, 851, p. 158301. Available at: https://doi.org/10.1016/j.scitotenv.2022.158301.

Dixit, A., Sahany, S. and Kulkarni, A.V. (2021) ‘Glacial changes over the Himalayan Beas basin under global warming’, *Journal of Environmental Management*, 295, p. 113101. Available at: https://doi.org/10.1016/j.jenvman.2021.113101.

Dolliver, H. and Gupta, S. (2008) ‘Antibiotic Losses in Leaching and Surface Runoff from Manure-Amended Agricultural Land’, *Journal of Environmental Quality*, 37(3), pp. 1227–1237. Available at: https://doi.org/10.2134/jeq2007.0392.

Dybka-Stępień, K., Antolak, H., Kmiotek, M., Piechota, D., and Koziróg, A. (2021) ‘Disposable Food Packaging and Serving Materials—Trends and Biodegradability’, *Polymers*, 13(20), p. 3606. Available at: https://doi.org/10.3390/polym13203606.

Eckert, E. M., Di Cesare, A., Coci, M., and Corno, G. (2018) ‘Persistence of antibiotic resistance genes in large subalpine lakes: the role of anthropogenic pollution and ecological interactions’, *Hydrobiologia*, 824(1), pp. 93–108. Available at: https://doi.org/10.1007/s10750-017-3480-0.

Frieri, M., Kumar, K. and Boutin, A. (2017) ‘Antibiotic resistance’, *Journal of Infection and Public Health*, 10(4), pp. 369–378. Available at: https://doi.org/10.1016/j.jiph.2016.08.007.

Geyer, R., Jambeck, J.R. and Law, K.L. (2017) ‘Production, use, and fate of all plastics ever made’, *Science Advances*, 3(7), p. e1700782. Available at: https://doi.org/10.1126/sciadv.1700782.

Goldenfeld, N. and Woese, C. (2007) ‘Biology’s next revolution’, *Nature*, 445(7126), pp. 369–369. Available at: https://doi.org/10.1038/445369a.

Haan, T.J. and Drown, D.M. (2021) ‘Unearthing Antibiotic Resistance Associated with Disturbance-Induced Permafrost Thaw in Interior Alaska’, *Microorganisms*, 9(1), p. 116. Available at: https://doi.org/10.3390/microorganisms9010116.

Harding, K. G., Dennis, J. S., von Blottnitz, H., and Harrison, S. T. L. (2007) ‘Environmental analysis of plastic production processes: Comparing petroleum-based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid using life cycle analysis’, *Journal of Biotechnology*, 130(1), pp. 57–66. Available at: https://doi.org/10.1016/j.jbiotec.2007.02.012.

Hellinger, W.C. and Brewer, N.S. (1999) ‘Carbapenems and monobactams: imipenem, meropenem, and aztreonam’, *Mayo Clinic Proceedings*, 74(4), pp. 420–434. Available at: https://doi.org/10.4065/74.4.420.

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., and Duflos, G. (2017) ‘Occurrence and effects of plastic additives on marine environments and organisms: A review’, *Chemosphere*, 182, pp. 781–793. Available at: https://doi.org/10.1016/j.chemosphere.2017.05.096.

Hirschberg, V. and Rodrigue, D. (2023) ‘Recycling of polyamides: Processes and conditions’, *Journal of Polymer Science*, 61(17), pp. 1937–1958. Available at: https://doi.org/10.1002/pol.20230154.

Hutchings, M.I., Truman, A.W. and Wilkinson, B. (2019) ‘Antibiotics: past, present and future’, *Current Opinion in Microbiology*, 51, pp. 72–80. Available at: https://doi.org/10.1016/j.mib.2019.10.008.

Jara, J., Alarcón, F., Monnappa, A. K., Santos, J. I., Bianco, V., Nie, P., Ciamarra, M. P., Canales, Á., Dinis, L., López-Montero, I., Valeriani, C., and Orgaz, B. (2021) ‘Self-Adaptation of Pseudomonas fluorescens Biofilms to Hydrodynamic Stress’, *Frontiers in Microbiology*, 11, p. 588884. Available at: https://doi.org/10.3389/fmicb.2020.588884.

Kaeberlein, T., Lewis, K. and Epstein, S.S. (2002) ‘Isolating “Uncultivable” Microorganisms in Pure Culture in a Simulated Natural Environment’, *Science*, 296(5570), pp. 1127–1129. Available at: https://doi.org/10.1126/science.1070633.

Kanhai, L. D. K., Gardfeldt, K., Krumpen, T., Thompson, R. C., and O'Connor, I. (2020) 'Microplastics in sea ice and seawater beneath ice floes from the Arctic Ocean', *Scientific Reports*, 10(1), p. 5004. Available at: https://doi.org/10.1038/s41598-020-61948-6.

Klemm, E.J., Wong, V.K. and Dougan, G. (2018) 'Emergence of dominant multidrug-resistant bacterial clades: Lessons from history and whole-genome sequencing', *Proceedings of the National Academy of Sciences*, 115(51), pp. 12872–12877. Available at: https://doi.org/10.1073/pnas.1717162115.

Kochtitzky, W. and Copland, L. (2022) 'Retreat of Northern Hemisphere Marine-Terminating Glaciers, 2000–2020', *Geophysical Research Letters*, 49(3), p. e2021GL096501. Available at: https://doi.org/10.1029/2021GL096501.

Kolář, M., Urbánek, K. and Látal, T. (2001) 'Antibiotic selective pressure and development of bacterial resistance', *International Journal of Antimicrobial Agents*, 17(5), pp. 357–363. Available at: https://doi.org/10.1016/S0924-8579(01)00317-X.

Kühn, S. and van Franeker, J.A. (2020) 'Quantitative overview of marine debris ingested by marine megafauna', *Marine Pollution Bulletin*, 151, p. 110858. Available at: https://doi.org/10.1016/j.marpolbul.2019.110858.

Laganà, P., Caruso, G., Corsi, I., Bergami, E., Venuti, V., Majolino, D., La Ferla, R., Azzaro, M., and Cappello, S. (2019) 'Do plastics serve as a possible vector for the spread of antibiotic resistance? First insights from bacteria associated to a polystyrene piece from King George Island (Antarctica)', *International Journal of Hygiene and Environmental Health*, 222(1), pp. 89–100. Available at: https://doi.org/10.1016/j.ijheh.2018.08.009.

Lamb, J. B., Willis, B. L., Fiorenza, E. A., Couch, C. S., Howard, R., Rader, D. N., True, J. D., Kelly, L. A., Ahmad, A., Jompa, J., and Harvell, C. D. (2018) 'Plastic waste associated with disease on coral reefs', *Science*, 359(6374), pp. 460–462. Available at: https://doi.org/10.1126/science.aar3320.

Larsson, D.G.J. (2014) 'Antibiotics in the environment', *Upsala Journal of Medical Sciences*, 119(2), pp. 108–112. Available at: https://doi.org/10.3109/03009734.2014.896438.

Lartaud, F., Meistertzheim, A. L., Reichert, J., Ziegler, M., Peru, E., and Ghiglione, J. F. (2020) 'Plastics: An Additional Threat for Coral Ecosystems', in, pp. 469–485. Available at: https://doi.org/10.1007/978-3-030-57054-5_14.

Lear, G., Kingsbury, J. M., Franchini, S., Gambarini, V., Maday, S. D. M., Wallbank, J. A., Weaver, L., and Pantos, O. (2021) 'Plastics and the microbiome: impacts and solutions', *Environmental Microbiome*, 16(1), p. 2. Available at: https://doi.org/10.1186/s40793-020-00371-w.

Lechthaler, S., Waldschläger, K., Stauch, G., and Schüttrumpf, H. (2020) 'The Way of Macroplastic through the Environment', *Environments*, 7(10), p. 73. Available at: https://doi.org/10.3390/environments7100073.

LeFrock, J.L., Prince, R.A. and Left, R.D. (1982) 'Mechanism of Action, Antimicrobial Activity, Pharmacology, Adverse Effects, and Clinical Efficacy of Cefotaxime', *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 2(4), pp. 174–184. Available at: https://doi.org/10.1002/j.1875-9114.1982.tb03185.x.

Lv, S., Li, Y., Zhao, S., and Shao, Z. (2024) 'Biodegradation of Typical Plastics: From Microbial Diversity to Metabolic Mechanisms', *International Journal of Molecular Sciences*, 25(1), p. 593. Available at: https://doi.org/10.3390/ijms25010593.

MacGowan, A. and Macnaughton, E. (2017) 'Antibiotic resistance', *Medicine*, 45(10), pp. 622–628. Available at: https://doi.org/10.1016/j.mpmed.2017.07.006.

MacLeod, M., Arp, H. P. H., Tekman, M. B., and Jahnke, A. (2021) 'The global threat from plastic pollution', *Science*, 373(6550), pp. 61–65. Available at: https://doi.org/10.1126/science.abg5433.

Manoharan, R. K., Srinivasan, S., Shanmugam, G., and Ahn, Y. H. (2021) 'Shotgun metagenomic analysis reveals the prevalence of antibiotic resistance genes and mobile genetic elements in full scale hospital wastewater treatment plants', *Journal of Environmental Management*, 296, p. 113270. Available at: https://doi.org/10.1016/j.jenvman.2021.113270.

Marathe, N.P. and Bank, M.S. (2022) 'The Microplastic-Antibiotic Resistance Connection', in M.S. Bank (ed.) *Microplastic in the Environment: Pattern and Process*. Cham: Springer

International Publishing, pp. 311–322. Available at: https://doi.org/10.1007/978-3-030-78627-4_9.

McCoy, L.S., Xie, Y. and Tor, Y. (2011) 'Antibiotics that target protein synthesis', *WIREs RNA*, 2(2), pp. 209–232. Available at: https://doi.org/10.1002/wrna.60.

Meng, J., Zhang, Q., Zheng, Y., He, G., and Shi, H. (2021) 'Plastic waste as the potential carriers of pathogens', *Current Opinion in Food Science*, 41, pp. 224–230. Available at: https://doi.org/10.1016/j.cofs.2021.04.016.

Microsoft Corporation, (2024). *Microsoft Excel*, Version (16.89) Available at: https://office.microsoft.com/excel.

Mindlin, S.Z., Soina, V.S., Petrova, M.A., & Gorlenko, Zh.M. (2008) 'Isolation of antibiotic resistance bacterial strains from Eastern Siberia permafrost sediments', *Russian Journal of Genetics*, 44(1), pp. 27–34. Available at: https://doi.org/10.1134/S1022795408010043.

Napper, I. E., Davies, B. F. R., Clifford, H., Elvin, S., Koldewey, H. J., Mayewski, P. A., Miner, K. R., Potocki, M., Elmore, A. C., Gajurel, A. P., and Thompson, R. C. (2020) 'Reaching New Heights in Plastic Pollution—Preliminary Findings of Microplastics on Mount Everest', *One Earth*, 3(5), pp. 621–630. Available at: https://doi.org/10.1016/j.oneear.2020.10.020.

Nathan Carl and Cars Otto (2014) 'Antibiotic Resistance — Problems, Progress, and Prospects', *New England Journal of Medicine*, 371(19), pp. 1761–1763. Available at: https://doi.org/10.1056/NEJMp1408040.

Neog, N., Phukan, U., Puzari, M., Sharma, M., and Chetia, P. (2021) 'Klebsiella oxytoca and Emerging Nosocomial Infections', *Current Microbiology*, 78(4), pp. 1115–1123. Available at: https://doi.org/10.1007/s00284-021-02402-2.

Ni, L., Xu, Y. and Chen, L. (2021) 'First Experimental Evidence for the Presence of Potentially Virulent Klebsiella oxytoca in 14 Species of Commonly Consumed Aquatic Animals, and Phenotyping and Genotyping of K. oxytoca Isolates', *Antibiotics*, 10(10), p. 1235. Available at: https://doi.org/10.3390/antibiotics10101235.

Nicolaou, K.C. and Rigol, S. (2018) ‘A brief history of antibiotics and select advances in their synthesis’, *The Journal of Antibiotics*, 71(2), pp. 153–184. Available at: https://doi.org/10.1038/ja.2017.62.

Oberbeckmann, S., Loeder, M. G. J., Gerdts, G., and Osborn, A. M. (2014) ‘Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters’, *FEMS Microbiology Ecology*, 90(2), pp. 478–492. Available at: https://doi.org/10.1111/1574-6941.12409.

Oberbeckmann, S., Kreikemeyer, B. and Labrenz, M. (2018) ‘Environmental Factors Support the Formation of Specific Bacterial Assemblages on Microplastics’, *Frontiers in Microbiology*, 8. Available at: https://doi.org/10.3389/fmicb.2017.02709.

Ofori, S., Puškáčová, A., Růžičková, I., and Wanner, J. (2021) ‘Treated wastewater reuse for irrigation: Pros and cons’, *Science of The Total Environment*, 760, p. 144026. Available at: https://doi.org/10.1016/j.scitotenv.2020.144026.

Olaitan, A. O., Diene, S. M., Kempf, M., Berrazeg, M., Bakour, S., Gupta, S. K., Thongmalayvong, B., Akkhavong, K., Somphavong, S., Paboriboune, P., Chaisiri, K., Komalamisra, C., Adelowo, O. O., Fagade, O. E., Banjo, O. A., Oke, A. J., Adler, A., Assous, M. V., Morand, S., Raoult, D., and Rolain, J. M. (2014) ‘Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study’, *International Journal of Antimicrobial Agents*, 44(6), pp. 500–507. Available at: https://doi.org/10.1016/j.ijantimicag.2014.07.020.

Ostle, C., Thompson, R. C., Broughton, D., Gregory, L., Wootton, M., and Johns, D. G. (2019) ‘The rise in ocean plastics evidenced from a 60-year time series’, *Nature Communications*, 10(1), p. 1622. Available at: https://doi.org/10.1038/s41467-019-09506-1.

Park, W.J., Hwangbo, M. and Chu, K.-H. (2023) ‘Plastisphere and microorganisms involved in polyurethane biodegradation’, *Science of The Total Environment*, 886, p. 163932. Available at: https://doi.org/10.1016/j.scitotenv.2023.163932.

Pérez-Vazquez, M., Oteo-Iglesias, J., Sola-Campoy, P. J., Carrizo-Manzoni, H., Bautista, V., Lara, N., Aracil, B., Alhambra, A., Martínez-Martínez, L., and Campos, J. (2019)

‘Characterization of Carbapenemase-Producing Klebsiella oxytoca in Spain, 2016–2017’, *Antimicrobial Agents and Chemotherapy*, 63(6), p. 10.1128/aac.02529-18. Available at: https://doi.org/10.1128/aac.02529-18.

Pinheiro, H.T., MacDonald, C., Santos, R.G., Ali, R., Bobat, A., Cresswell, B.J., Francini-Filho, R., Freitas, R., Galbraith, G.F., Musembi, P., Phelps, T.A., Quimbayo, J.P., Quiros, T.E.A.L., Shepherd, B., Stefanoudis, P.V., Talma, S., Teixeira, J.B., Woodall, L.C., & Rocha, L.A. (2023) ‘Plastic pollution on the world’s coral reefs’, *Nature*, 619(7969), pp. 311–316. Available at: https://doi.org/10.1038/s41586-023-06113-5.

Pirahanchi, Y. and Sharma, S. (2024) ‘Biochemistry, Lipase’, in *StatPearls*. Treasure Island (FL): StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/books/NBK537346/ (Accessed: 19 September 2024).

Pontes, M.H. and Groisman, E.A. (2020) ‘A Physiological Basis for Nonheritable Antibiotic Resistance’, *mBio*, 11(3), p. 10.1128/mbio.00817-20. Available at: https://doi.org/10.1128/mbio.00817-20.

Provencher, J. F., Ammendolia, J., Rochman, C. M., and Mallory, M. L. (2019) ‘Assessing plastic debris in aquatic food webs: what we know and don’t know about uptake and trophic transfer’, *Environmental Reviews*, 27(3), pp. 304–317. Available at: https://doi.org/10.1139/er-2018-0079.

Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J., and Segata, N. (2017) ‘Shotgun metagenomics, from sampling to analysis’, *Nature Biotechnology*, 35(9), pp. 833–844. Available at: https://doi.org/10.1038/nbt.3935.

Radisic, V., Nimje, P. S., Bienfait, A. M., and Marathe, N. P. (2020) ‘Marine Plastics from Norwegian West Coast Carry Potentially Virulent Fish Pathogens and Opportunistic Human Pathogens Harboring New Variants of Antibiotic Resistance Genes’, *Microorganisms*, 8(8), p. 1200. Available at: https://doi.org/10.3390/microorganisms8081200.

Radisic, V., Victor, M. P., Grevskott, D. H., and Marathe, N. P. (2024) ‘Novel antibiotic resistance genes from the hospital effluent are disseminated into the marine environment in Norway’. medRxiv, p. 2024.09.23.24313887. Available at: https://doi.org/10.1101/2024.09.23.24313887.

Richards, Z.T. and Beger, M. (2011) ‘A quantification of the standing stock of macro-debris in Majuro lagoon and its effect on hard coral communities’, *Marine Pollution Bulletin*, 62(8), pp. 1693–1701. Available at: https://doi.org/10.1016/j.marpolbul.2011.06.003.

RStudio Team (2022). RStudio: Integrated Development Environment for R. Version (2022.12.0+353), Boston, MA. Available at: http://www.rstudio.com/.

Salazar, A., Rico, A., Rodríguez, J., Escudero, J. S., Seltzer, R., and Martin de la Escalera Cutillas, F. (2014) ‘Monotonic loading and fatigue response of a bio-based polyamide PA11 and a petrol-based polyamide PA12 manufactured by selective laser sintering’, *European Polymer Journal*, 59, pp. 36–45. Available at: https://doi.org/10.1016/j.eurpolymj.2014.07.016.

Santos, J.A. and Lamers, M.H. (2020) ‘Novel Antibiotics Targeting Bacterial Replicative DNA Polymerases’, *Antibiotics*, 9(11), p. 776. Available at: https://doi.org/10.3390/antibiotics9110776.

Schwarz, A. E., Ligthart, T. N., Boukris, E., and van Harmelen, T. (2019) ‘Sources, transport, and accumulation of different types of plastic litter in aquatic environments: A review study’, *Marine Pollution Bulletin*, 143, pp. 92–100. Available at: https://doi.org/10.1016/j.marpolbul.2019.04.029.

Sérvulo, T., Taylor, J. D., Proietti, M. C., Rodrigues, L. d. S., Puertas, I. P., Barutot, R. A., and Lacerda, A. L. d. F. (2023) ‘Plastisphere composition in a subtropical estuary: Influence of season, incubation time and polymer type on plastic biofouling’, *Environmental Pollution*, 332, p. 121873. Available at: https://doi.org/10.1016/j.envpol.2023.121873.

Sharma, S.K., Singh, L. and Singh, S. (no date) ‘Comparative Study between Penicillin and Ampicillin’.

Sheridan, E.A., Fonvielle, J.A., Cottingham, S., Zhang, Y., Dittmar, T., Aldridge, D.C., & Tanentzap, A.J. (2022) ‘Plastic pollution fosters more microbial growth in lakes than natural organic matter’, *Nature Communications*, 13(1), p. 4175. Available at: https://doi.org/10.1038/s41467-022-31691-9.

Singh, L., Cariappa, M.P. and Kaur, M. (2016) ‘Klebsiella oxytoca: An emerging pathogen?’, *Medical Journal, Armed Forces India*, 72(Suppl 1), pp. S59–S61. Available at: https://doi.org/10.1016/j.mjafi.2016.05.002.

Soucy, S.M., Huang, J. and Gogarten, J.P. (2015) ‘Horizontal gene transfer: building the web of life’, *Nature Reviews Genetics*, 16(8), pp. 472–482. Available at: https://doi.org/10.1038/nrg3962.

Spiers, A.J., Buckling, A. and Rainey, P.B. (2000) ‘The causes of Pseudomonas diversity’, *Microbiology*, 146(10), pp. 2345–2350. Available at: https://doi.org/10.1099/00221287-146-10-2345.

Stanier, R.Y., Palleroni, N.J. and Doudoroff, M. (1966) ‘The Aerobic Pseudomonads a Taxonomic Study’, *Microbiology*, 43(2), pp. 159–271. Available at: https://doi.org/10.1099/00221287-43-2-159.

Stevenson, E.M., Buckling, A., Cole, M., Lindeque, P.K., & Murray, A.K. (2024) ‘Selection for antimicrobial resistance in the plastisphere’, *Science of The Total Environment*, 908, p. 168234. Available at: https://doi.org/10.1016/j.scitotenv.2023.168234.

Stewart, E.J. (2012) ‘Growing Unculturable Bacteria’, *Journal of Bacteriology*, 194(16), pp. 4151–4160. Available at: https://doi.org/10.1128/jb.00345-12.

Sullivan, G.J., Delgado, N.N., Maharjan, R., & Cain, A.K. (2020) ‘How antibiotics work together: molecular mechanisms behind combination therapy’, *Current Opinion in Microbiology*, 57, pp. 31–40. Available at: https://doi.org/10.1016/j.mib.2020.05.012.

Sun, X., Chen, B., Xia, B., Li, Q., Zhu, L., Zhao, X., Gao, Y., & Qu, K. (2020) ‘Impact of mariculture-derived microplastics on bacterial biofilm formation and their potential threat to mariculture: A case *in situ* study on the Sungo Bay, China’, *Environmental Pollution*, 262, p. 114336. Available at: https://doi.org/10.1016/j.envpol.2020.114336.

Tang, K.W.K., Millar, B.C. and Moore, J.E. (2023) ‘Antimicrobial Resistance (AMR)’, *British Journal of Biomedical Science*, 80, p. 11387. Available at: https://doi.org/10.3389/bjbs.2023.11387.

Thushari, G.G.N. and Senevirathna, J.D.M. (2020) 'Plastic pollution in the marine environment', *Heliyon*, 6(8), p. e04709. Available at: https://doi.org/10.1016/j.heliyon.2020.e04709.

Tourova, T., Sokolova, D.S., Nazina, T.N., & Grouzdev, D. (2020) 'Biodiversity of Microorganisms Colonizing the Surface of Polystyrene Samples Exposed to Different Aqueous Environments', *Sustainability*, 12(9), p. 3624. Available at: https://doi.org/10.3390/su12093624.

Vasanthan, N. (2009) 'Polyamide fiber formation: Structure, properties and characterization', in *Handbook of Textile Fibre Structure*, pp. 232–256. Available at: https://doi.org/10.1533/9781845696504.2.232.

Vranakis, I., Goniotakis, I., Psaroulaki, A., Sandalakis, V., Tselentis, Y., Gevaert, K., & Tsiotis, G. (2014) 'Proteome studies of bacterial antibiotic resistance mechanisms', *Journal of Proteomics*, 97, pp. 88–99. Available at: https://doi.org/10.1016/j.jprot.2013.10.027.

Weinstein, J.E. *et al.* (2020) 'Degradation of bio-based and biodegradable plastics in a salt marsh habitat: Another potential source of microplastics in coastal waters', *Marine Pollution Bulletin*, 160, p. 111518. Available at: https://doi.org/10.1016/j.marpolbul.2020.111518.

Worm, B., Lotze, H.K., Jubinville, I., & Wilcox, C. (2017) 'Plastic as a Persistent Marine Pollutant', *Annual Review of Environment and Resources*, 42(1), pp. 1–26. Available at: https://doi.org/10.1146/annurev-environ-102016-060700.

Yang, J., Long, H., Hu, Y., Feng, Y., McNally, A., & Zong, Z. (2022) 'Klebsiella oxytoca Complex: Update on Taxonomy, Antimicrobial Resistance, and Virulence', *Clinical Microbiology Reviews*, 35(1), pp. e00006-21. Available at: https://doi.org/10.1128/CMR.00006-21.

Yildizhan, F.S. (2021) 'A Technical and Industrial Analysis of Global Plastics Market, Trade, Financing, and Operations', *ScienceOpen Preprints* [Preprint]. Available at: https://doi.org/10.14293/S2199-1006.1.SOR-.PPDUNRN.v1.

Zettler, E.R., Mincer, T.J. and Amaral-Zettler, L.A. (2013) 'Life in the "Plastisphere": Microbial Communities on Plastic Marine Debris', *Environmental Science & Technology*, 47(13), pp. 7137–7146. Available at: https://doi.org/10.1021/es401288x.

Zhai, X., Zhang, X.-H. and Yu, M. (2023) ‘Microbial colonization and degradation of marine microplastics in the plastisphere: A review’, *Frontiers in Microbiology*, 14, p. 1127308. Available at: https://doi.org/10.3389/fmicb.2023.1127308.

Zhu, D., Ma, J., Li, G., Rillig, M.C., & Zhu, Y.-G. (2022) ‘Soil plastispheres as hotspots of antibiotic resistance genes and potential pathogens’, *The ISME Journal*, 16(2), pp. 521–532. Available at: https://doi.org/10.1038/s41396-021-01103-9.