Double trouble: plastic leachates as drivers for increased adaptations to antibiotics?

The effects of plastic leachates and ciprofloxacin on the sea urchin microbiome.

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Thesis submitted for the degree of Master of Science in Toxicology and Environmental Science

60 credits

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May 2022

2022

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Abstract

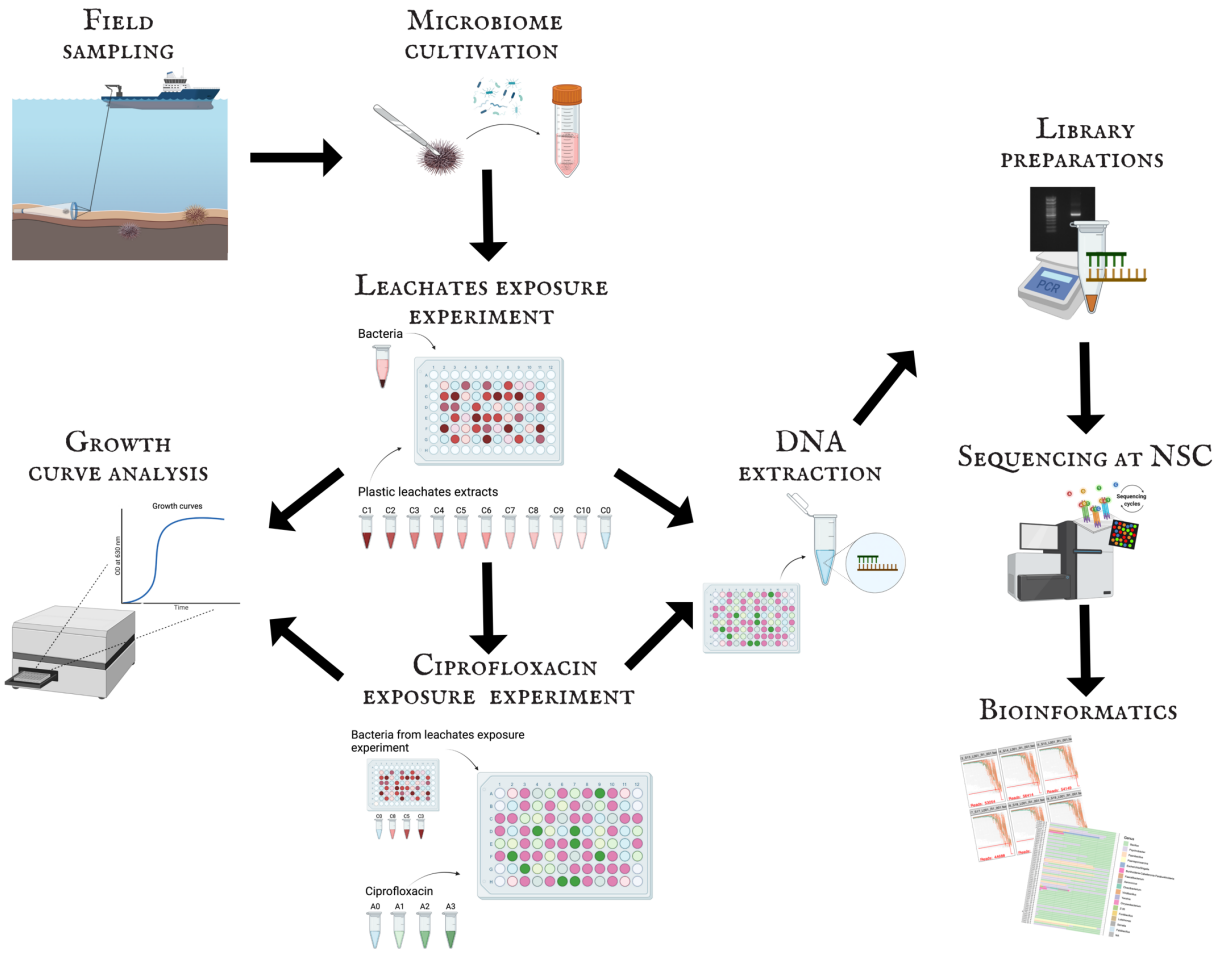
Additives leaching out of plastics may affect the surrounding environment and are thus of environmental concern. Additionally, the potential for cross-resistance to antibiotics following plastic exposure in bacteria raises concern, as a variety of toxic substances originating from plastic additives may induce efflux pumps providing resistance against both leaching toxicants and antibiotics. The aim of this study was to explore the effects of plastic leachates on bacterial communities originating from a marine environment and assess whether bacterial communities preconditioned with plastic leachates obtained increased adaptations to antibiotics.

The microbiome of the marine, sediment dwelling sea urchin *Brissopsis lyrifera* was enriched and subsequently inoculated with ten different concentrations of leachates extracted from dishwashing gloves, ranging from 99.2 µg/L to 1.94 g/L. Following leachate exposure, four selected inoculates, representing different leachate concentrations, were exposed to three ciprofloxacin concentrations ranging from 25 ng/L to 25 mg/L. Bacterial growth curves were obtained by measuring optical density at 630 nm, and bacterial community composition was assessed using DNA metabarcoding targeting the 16S rRNA gene using Illumina gene sequencing.

Decreased maximum growth rates and yield, prolonged lag phases and increased alpha diversity were observed at the highest tested concentrations of leachates. Although inconclusive effects were observed in beta diversity, eight bacterial genera were significantly affected by leachates. Preconditioned enrichments were negatively affected by ciprofloxacin as observed through decreased maximum growth rates, however no significant interaction effects of leachates and ciprofloxacin exposure were observed in alpha diversity. Leachates and ciprofloxacin induced log-fold changes in three bacterial genera.

No increased community adaptation to ciprofloxacin following preconditioning with leachates could be observed in the enriched microbiome. On the contrary the results point toward potential adverse effects of the interaction between those substances when present simultaneously in a mixture. Furthermore, our results add to the increasing literature on plastic leachate's adverse effects on microbiomes associated with marine life with the potential to induce functional alterations of marine ecosystems.

Graphical abstract



Til pappa

Acknowledgments

There are many people who have contributed to this master thesis, and without whom this project would never have seen the light of day. To my main supervisor, Alexander Eiler (UiO), thank you for all your help, discussions, laughs and experiences in the lab and field that made these past two years be so much more than merely a time of study. Your guidance has been invaluable to me. To my co-supervisor Ketil Hylland (UiO), thank you for all the possibilities you have given me during these years; being able to teach and help others while learning has been a great privilege. I have really appreciated working with you, and the many hours at the lab in Drøbak and at Trygve Braarud have been a lot of fun thanks to you. To my other co-supervisor Tânia Gomes (NIVA), thank you for kind and helpful guidance in manoeuvring the world of plastics. This work could not have been done without the plastic leachates, so I am very grateful for being able to use them in this project thanks to Lisbet Sørensen and Andy M. Booth at the Department of Environment and New Resources, SINTEF Ocean, Trondheim, in collaboration with Tânia.

There are many people I met at the Department of Biosciences that I wish to thank, who have contributed so kindly when I needed help. Sverre, your shared experiences have been the backbone of this project. Jing, I really appreciate all your help in the lab, and all our adventures in the labyrinth that is IBV. Berit and Per-Johan, your help saved my experiments on more than one occasion. Pedro and Laurent, your help with the library preparations were much appreciated, and Bård, Athanasios and Amar, thank you for guidance and help in choosing and locating equipment. Tom, thank you for your valuable help and insight into statistics – I would have been very confused still, had it not been for your help.

Eivind and Even, you deserve a special thanks. Without you, not only would this thesis never have happened, but it wouldn't have been half as fun. I think we have spent more time together the past two years than anyone else I know, and it has been great. It is safe to say that we challenged corona together – and won! And a big shout-out to vb reading room (4405) and especially Hanne (lab partners for life) and Eli. You truly are Good girls.

Finally, to my family, Mamma, Pappa, Oda, Ekko and Kasper. Your endless support has been vital in this work. Pappa, you saw the start of this project, but unfortunately never witnessed the ending, but know that you are with me in every step I take in academia. My future is because of you.

Eira Catharine Lødrup Carlsen

Oslo, May 2022

Abbreviations

16S rRNA – 16S ribosomal RNA

3,5 DCP – 3,5 Dichlorophenyl

ACE – Abundance-based coverage estimators

Adj. – Adjusted p-value

ADOC – Anthropogenic dissolved organic carbon

ANOVA – Analysis of Variance

ASV – Amplicon sequence variant

BPA – Bisphenol A

CI – Confidence interval

DCM - Dichloromethane

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EC50 – Half maximal effective concentration

ECHA – European chemical agency

Eta2 – Eta squared (effect size)

EtAc - Ethyl acetate

GC-MS – Gas chromatography -mass spectrometry

Glm – Generalised linear models

IBV – Department of Biosciences

MIC – Minimum inhibitory concentration

NIST – National Institute of Standards and Technology

NIVA – Norwegian Institute for Water Research

NMDS – Non-metric Multi-dimentional Scaling

NSC – Norwegian Sequencing Centre

OD – Optical density

OD/h – Optical density change per hour

OECD – Organisation for Economic Co-operation and Development

p.significant – The significance level of the p-value

PAH – Polyaromatic hydrocarbons

PBDE – Polybrominated diphenyl ethers

PCB – Polychlorinated biphenyls

PCR – Polymerase chain reaction

PERMANOVA – Permutational analysis of variance

R2 – R squared (coefficient of determination)

Rlog – Regularised logarithm

RNA – Ribonucleic acid

Rpm – Rounds per minutes

Std.beta – Standardised beta

UiO – University of Oslo

YCFA – Yeast Casitone Fatty Acid medium

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1 Introduction

1.1 Plastic (not so fantastic) leachates

Plastic products have since the beginning of its production become widespread and are essential in many aspects of daily life. The total amount of plastics produced by 2021 was 9,2 billion tons (UNEP, 2021), and in 2020 alone, the amount of plastic produced (not including the recycled plastic production) was quantified to 367 million tons (PlasticsEurope, 2021), which compared to the 1,7 million tons produced in 1950 is a comprehensive increase (PlasticsEurope, 2013). The production is estimated to increase significantly in the years to come (Delaeter et al., 2022; Zalasiewicz et al., 2016). With production comes waste, and the amount of plastic waste in 2015 was 4900 metric tons (Geyer et al., 2017). In 2016, 11 million tons of plastic ended up in our oceans, a number which is expected to be more than double in the years to come (Jambeck et al., 2015; UNEP, 2021). Consequently, plastics are a large part of the anthropogenic footprint on the environment, and have been found worldwide, even at remote places such as in the Arctic (Eriksen et al., 2014; Nelms et al., 2017; Obbard et al., 2014; Zalasiewicz et al., 2016).

The properties of plastics may cause major concern, as they are inert, thus difficult to degrade and persistent in the environment, have low density and are mobile, and can thus be transported over long distances in the water and by wind (Amelia et al., 2021; Delaeter et al., 2022; Galloway, 2015; Lithner et al., 2011; Nelms et al., 2017; Zalasiewicz et al., 2016). Microplastics defined as particles below 5 mm (ECHA, 2022d), originating from microplastics production or physical and or physicochemical degradation of larger plastics, are mobile and have been identified in marine waters, sediments (Zalasiewicz et al., 2016), and recently in human blood (Leslie et al., 2022). Thus, the presence of plastic and microplastic in diverse biological and environmental matrices may pose serious threats to the health of ecosystems, and a growing concern for the environment and human well-being have been raised as consequence to plastic pollution (Amelia et al., 2021; Galloway, 2015; Ivleva et al., 2017; Lambert & Wagner, 2018).

Plastics consist of either fully synthetic polymers or polymers originating from cellulose and petrochemicals (Zalasiewicz et al., 2016). In addition, several chemicals are added to the polymers to create the desired properties of the plastic (Hermabessiere et al., 2017; Lithner et al., 2011; OECD, 2014). For instance antioxidants and preservatives, including bacteriostatics, function as stabilisers to extend the plastics life (OECD, 2014). The chemicals used in plastic manufacturing, referred to as additives, have different origin, structure, and function (Gunaalan et al., 2020; Lithner et al., 2011). Per today over 400 harmful chemicals are used as plastic additives, as identified by the European Chemicals Agency (ECHA) plastic additives initiative (ECHA, 2018). Such additives may leach out of the plastic product, either

when heated (Talsness et al., 2009) or during degradation of the plastics in the environment (Bejgarn et al., 2015; Gunaalan et al., 2020; Luo et al., 2019; Zhang et al., 2019), and affect organisms in the surrounding environment (Bejgarn et al., 2015; Delaeter et al., 2022; Li et al., 2016; Lithner et al., 2009; Luo et al., 2019; Martínez-Gómez et al., 2017; Seuront, 2018).

The known harmful additives include brominated flame retardants, phthalates, nonylphenols and bisphenol A (BPA) (Hermabessiere et al., 2017; Lithner et al., 2011). Environmental concentrations of some of the most common plastic additives, such as BPA, polybrominated diphenyl ethers (PBDE) and phthalates, have been found in concentrations up to µg/L in marine waters and in ranges from ng/kg – mg/kg in sediments (Hermabessiere et al., 2017). Some of the adverse effects observed in microorganisms include inhibition of growth, decrease in photosynthetic activity, altered membrane integrity and genotoxic effects (Alabi et al., 2019; Capolupo et al., 2020; Hjelset, 2021; Schiavo et al., 2021; Tetu et al., 2020; Tetu et al., 2019). In addition to plastic additives intentionally added, chemicals may adsorb onto the plastic surface (Hirai et al., 2011; Teuten et al., 2009). Chemicals identified as being adsorbed onto the plastic surface includes chemicals of environmental concerns, such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) (Hirai et al., 2011; Teuten et al., 2009), trace metals, such as zinc and copper (Brennecke et al., 2016) and antibiotics (Li et al., 2018).

1.2 Bacterial adaptations to plastics and antibiotic resistance

Being directly exposed to the environment surrounding it, microorganisms have developed different ways to cope with chemicals in the environment causing adverse effects (Nikaido, 2001). While being effective against many chemicals, the outer membrane of gram-negative bacteria and some gram-positive bacteria do not prevent all chemicals from entering the cells. Thus the bacteria also have a system of transporting these chemicals out of the cell, by the means of efflux pumps (Nikaido, 2001). These efflux pumps have a wide substrate range spanning from solvents and dyes to trace metals and antibiotics (Martinez et al., 2009; Nikaido, 2001; Nishino et al., 2007; Papkou et al., 2020; Poole, 2000; Silver & Phung, 1996) with different degrees of specificity (Hernández et al., 1998; Imran et al., 2019; Martinez et al., 2009; Nishino et al., 2007; Perron et al., 2004; Webber & Piddock, 2003). Bacteria exposed to plastic leachates have been shown to respond to the leachates by increased transcription of efflux pumps (Tetu et al., 2019), which may induce cross-resistance towards antibiotics. In addition, metals have been observed in plastic leachates (Brennecke et al., 2016; Cheng et al., 2010; Groh et al., 2019; Hahladakis et al., 2018) and therefore the exposure to plastic leachates containing metals may induce increased transcription and expression of efflux pumps, and thus promote increased adaptations towards antibiotics. Antibiotic resistant genes and antibiotics present on microplastics (Du et al., 2022; Imran et al., 2019; Yang et al.,

2019; Zhang et al., 2019) may further induce selection of antibiotic resistant bacteria and represent an underscored global concern.

A common fluoroquinolone antibiotic, ciprofloxacin has been used extensively due to its wide spectrum of activity and is thought to have led to a high degree of quinolone resistant bacteria (Agnello & Wong-Beringer, 2012; Poole, 2000; Redgrave et al., 2014; Ruiz, 2003; Sharma et al., 2010). One of the mechanisms of which bacteria become resistant to ciprofloxacin is through induced efflux pumps (Oethinger et al., 2000; Poole, 2000; Redgrave et al., 2014; Ricci et al., 2004; Ricci et al., 2006; Webber & Piddock, 2003).

1.3 The importance of being a bioturbator

Many organisms may encounter and mistake microplastic particles for food particles (Franzellitti et al., 2019; Gunaalan et al., 2020; Setälä et al., 2016). In addition to having potential adverse impacts resulting from malnutrition, such ingestion can lead to accumulation of microplastics in the gut (Franzellitti et al., 2019; Gunaalan et al., 2020). Sediments are considered to be sinks of microplastics, with microplastics identified in sediments as deep as 60 cm (Xue et al., 2020). Thus, sediment-dwelling invertebrates are at risk of exposure to plastic particles. The gut microbiome of the sediment dwelling invertebrates may thus be exposed to plastic particles, and with them, their leachates.

A common sediment-dwelling invertebrate in the Oslofjord is the sea urchin *Brissopsis lyrifera*. Being an infaunal deposit feeder, *B. lyrifera* feed non-selectively on organic particles adhering to sediment particles (Hollertz & Duchêne, 2001) in the sediment surface and subsurface (Austen & Widdicombe, 1998). Through burrowing sediments in depths up to 10 cm (Widdicombe et al., 2004), most of their bioturbation is caused by reworking of the sediments through movement, although some of the bioturbation are the result of ingestion (Hollertz & Duchêne, 2001). Bioturbation by *B. lyrifera* is an important ecosystem service, as it has a positive impact on species diversity (Widdicombe et al., 2004), maintains regional diversity (Widdicombe & Austen, 1998), alters sediment particle size distribution, and facilitates increased respiration, as well as nutrient and oxygen distribution in deeper sediments (Austen & Widdicombe, 1998; Hollertz & Duchêne, 2001; Widdicombe & Austen, 1998). The species has a significant impact on biogeochemical processes in the sediments (Hollertz & Duchêne, 2001).

Some initial studies have suggested that pollution can affect the microbiome of marine invertebrates; Hochstein et al. (2019) showed that the gut microbiome of the marine polychaete *Capitella teleta* experienced an increase in genes involved with chemoheterotrophy when exposed to hydrocarbon pollution. Milan et al. (2018) showed that pollution affecting benthic microbiomes may impact their hosts interactions with the environment. In addition, resistance towards xenobiotics have been identified in the microbiome of Antarctic sea urchins (González-Aravena et al., 2016). As such, cross-resistance

involving antibiotics may occur in the environment, and thus, exposure to pollution may impact both the microbiome and its host, possibly affecting the host physiology with possible implications for the ecosystem services performed by these organisms.

In order to observe the potential susceptibility of bacterial communities towards antibiotics in association with plastic leachates on bacterial communities, we exposed the enriched microbiome of a sediment-dwelling sea urchin sampled from the Oslofjord to plastic leachates from dishwashing gloves, before exposing them to ciprofloxacin.

1.4 Objectives and aims

In this project, the microbiome of *B. lyrifera* was used to assess potential community adaptation towards antibiotics in the presence of plastic leachates in bacterial communities belonging to environmentally significant host organisms. The overall objective of this thesis was thus to explore the effects of leachates and antibiotics on bacterial communities.

Firstly, we hypothesise that leachates from dishwasher gloves will induce adverse effects on the cultivable gut microbiome of the marine, sediment dwelling invertebrate *Brissopsis lyrifera*. The specific aims were therefore to explore if:

1) growth parameters such as maximum growth rate and yield were reduced, and the lag phases prolonged with increased exposure to leachates

2) bacterial community diversity and composition changed with increased leachate exposure measured through alpha and beta-diversity

3) increased leachate concentrations induced log fold changes in bacterial genera

Secondly, we hypothesise that prior exposure to leachates will lead to a decreased community susceptibility to antibiotics using ciprofloxacin as a model. The specific aims were therefore to explore if:

1) bacterial communities with, compared to without leachate preconditioning had increased yield and maximum growth rates, and reduced duration of lag phases under ciprofloxacin exposure

2) bacterial communities with, compared to without leachate preconditioning had changes in bacterial community diversity and composition under ciprofloxacin exposure measured through alpha and beta-diversity

3) bacterial communities with, compared to without leachate preconditioning had log fold changes in bacterial genera under ciprofloxacin exposure

2 Materials and methods

2.1 Field sampling

The sampling was conducted over a period of two days, on the 4th and 5th of March 2021 in the outer Oslofjord by Drøbak, south of Storkjær (59°39'19"N, 10°36'27"E)(figure 1).

Figure 1. Map over the Oslofjord. The sampling site, in the Oslofjord outside of Drøbak, is marked with a red pin. Map is collected from © norgeskart.no.

An Agassiz trawl was used to sample benthic species at 60-70 metres depth. The animals were collected from the sediments and kept in buckets with fresh seawater to keep them alive until processing. They were rinsed well in seawater to remove sediments from the animal surface. The gut of the sea urchin *Brissopsis lyrifera* (figure 2) was dissected out, inoculated with modified Yeast Casitone Fatty Acid (YCFA) medium prepared days before (see the appendix for medium preparation protocol) and snap frozen in liquid nitrogen before being stored at -80 °C until further processing. The medium was chosen based on its capacity to enrich diverse gut microbiomes.

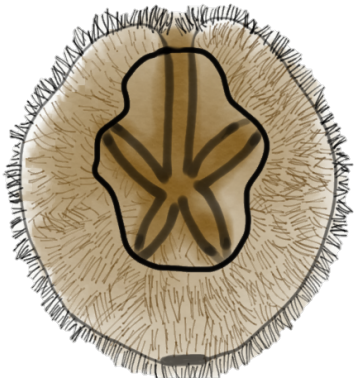

Figure 2. Illustration of the sea urchin Brissopsis lyrifera.

2.2 Enrichment preparations

To obtain the bacterial community to be used in the *in vitro* toxicity testing of leachates and ciprofloxacin, the dissected gut of the heart urchin was homogenised using pestle and mortar. The homogenate was inoculated in a modified YCFA medium for bacterial growth (see figure 3 for illustration). The inoculation was stored at 7-12 ºC on a rocking table with horizontal rocking movements to prevent anoxic zones in the inoculate. The inoculate was kept in this way for eight days, until signs of microbial growth such as a black line was visible in the surface of the medium, and the medium became turbid. When bacterial growth had occurred, 200 µl of the inoculate was transferred to new, sterile vials and mixed with approximately 100 µl 100% glycerol (Merck Life Science AS/ Sigma Aldrich Norway AS). The samples were then immediately frozen at -20ºC until further processing. Glycerol was chosen to improve the survival of as many bacteria as possible when frozen.

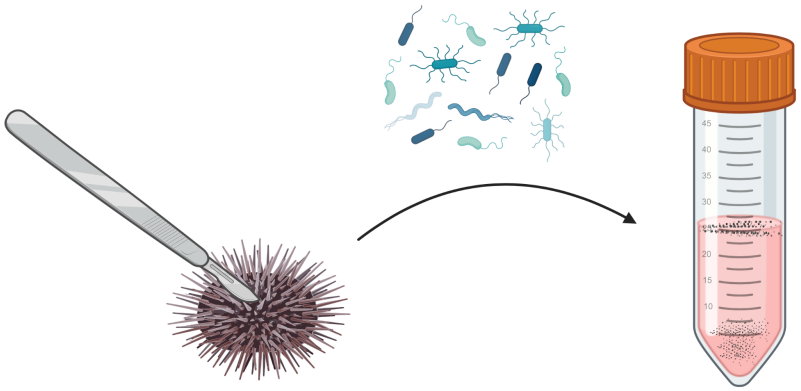

Figure 3. Illustration of enrichment preparations. The microbiome of the heart urchin was dissected out and incubated in YCFA medium. Illustration was made using Biorender.com.

2.3 Leachates exposure experiment

2.3.1 Plastic leachates preparations

All plastic leachate preparations and chemical analysis were performed according to the methods used by Zimmermann et al (2019), under the Project MicroLEACH (Grant 295174) funded by the Norwegian Research Council. The plastic leachates in this experiment were extracted from commercially sourced washing gloves (figure 4). Prior to use, the washing gloves were cut into small pieces (< 1 cm in all dimensions). Thus, the leachates in this experiment are primary leachates, a concept suggested by Huang et al. (2021). To identify the chemicals present, samples of leachates (100 mg) were extracted using DCM and EtAc (1:1, v/v) (n=3), before undergoing a non-target screening of the chemical content. This was done using gas chromatography-mass spectrometry (GC-MS) analysis, by using an Agilent 7890A GC coupled to an Agilent 5975 C MS. To identify the chemicals, peaks were identified in spectra that had been deconvoluted, and subsequently compared to NIST reference libraries. The identifications are tentative, with a higher % match as indicator of higher certainty. See appendix for an overview of the chemicals present in the leachates.

Figure 4. Picture of the plastic gloves used to extract leachates. Foto: Tânia Gomes.

2.3.2 Plastic leachate exposure experiment

The different concentrations of leachates were prepared by diluting the leachates with 100% dimethyl sulfoxide (DMSO). The concentrations were based on the preliminary EC50 results for the microalgae *Skeletonema pseudocostatum* exposed for 72 hours (0.0031 mg/mL) to the

leachates (Gomes et al., unpublished data). This concentration was kept as the middle concentration, with a concentration range from 4.96 ng/mL to 1.9375 mg/mL (see table 1). An updated EC50 of 0.003682 ± 0.0000014 mg plastic/mL was received in May 2022, based on the results from two independent experiments with *S. pseudocostatum* (Gomes et al., unpublished data).

Table 1. Concentrations of plastic leachates exposed to bacterial enrichments.

Treatment ID	Final concentration of leachates (mg/mL)
C1	1.9375
C2	0.3875
C3	0.0775
C4	0.0155
C5	0.0031
C6	0.00062
C7	0.000124
C8	0.0000248
C9	0.00000496
C10	0.000000992
C0	0

3,5 dichlorophenol (3,5 DCP) was used as positive control as in Zimmermann et al. (2019) at a concentration of 0.0066 mg/mL. See appendix for overview of the content of each sample. The enriched bacteria were thawed, and then inoculated (1 µl) with fresh modified YCFA medium (194 µl) and the different concentrations of leachates (5 µl) in separate wells in a sterile 96 well microtiter plate. The samples were distributed randomly in the 96 wells using the "*wpm"* package in R (Borges et al., 2021), but avoiding the edges to avoid possible edge effects (see figure 5 for illustration). The plate was covered with sterile sealing tape, allowing oxygen to enter the wells but preventing contamination from the surrounding environment. The plate was immediately inserted in a microplate reader (Synergy™ MX), and measurements of absorbance at 630 nm was initiated. Measurements were made every 30 minutes until all samples reached the stationary phase. This lasted for 97.5 hours. Prior to each measurement, medium shaking for 10 seconds was performed to prevent bacteria from adhering to the wall of the wells. After the 97.5 hours, the ciprofloxacin exposure was initiated immediately, involving sampling 1 µl of selected samples to the ciprofloxacin exposure experiment. Thereafter, the samples were transferred to a new plate without flat bottoms and centrifuged in a tabletop centrifuge at 4000 rounds per minute (rpm) for 10 minutes at 5°C, to obtain a bacterial pellet in each well. The supernatant of each well was discarded, and the bacterial pellet was resuspended in 180 µl enzymatic lysis buffer. The plate was covered with film, and immediately frozen at -20 °C until DNA extraction.

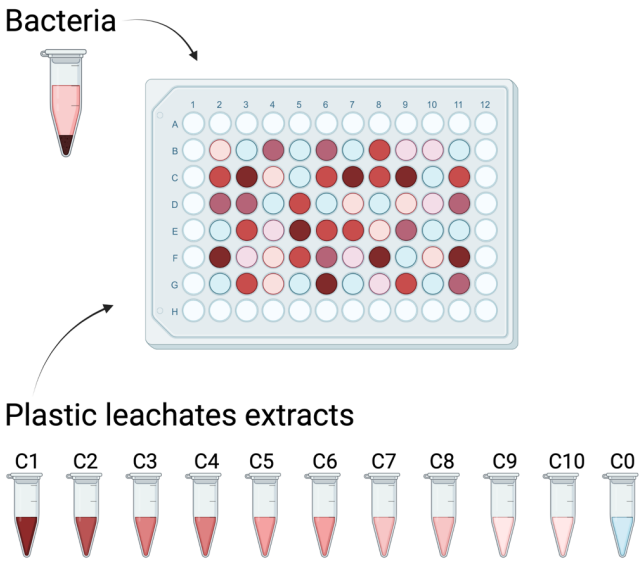

Figure 5. Illustration of the leachate exposure experiment. Enriched bacteria were inoculated in a 96 well plate with ten different concentrations of leachates, before optical density measures at 630 nm were made. Illustration was made using Biorender.com.

2.4 Ciprofloxacin exposure experiment

2.4.1 Ciprofloxacin stock solution preparations

The ciprofloxacin concentrations were prepared by mixing ciprofloxacin stock powder (Merck Life Science AS/ Sigma Aldrich Norway AS) with 1% acetic acid. The antibiotic was filter sterilised using a 0.22 µm filter. The concentrations of ciprofloxacin were chosen based on minimal inhibition concentrations (MICs) (The European Committee on Antimicrobial Susceptibility Testing, 2021) and concentrations found in the environment, to keep the experiment environmentally relevant (Grenni et al., 2018). Three ciprofloxacin concentrations were used: 25 ng/L, 2.5 µg/L and 0.25 mg/L. See appendix for overview of the content of all samples.

2.4.2 Ciprofloxacin exposure experiment

Immediately after the leachate exposure experiment ended, the ciprofloxacin exposure experiment was initiated. Modified YCFA medium was added to each well in a new, sterile 96 well microtiter plate. Then 1 µl of the bacteria from the selected leachate concentrations from the leachate exposure (table 2) was transferred to a new plate, followed by inoculation with ciprofloxacin (figure 6). As in the first experiment, the samples were distributed randomly, and absorbance measurements were made every 30 minutes using the same settings as in the first experiment. Stationary phase was reached in almost all wells after 44.5 hours. When ending

the measurements, the plate was treated as in the first experiment (resuspending bacterial pellet in enzymatic lysis buffer) and stored at -20ºC Celsius.

Table 2: Concentrations of plastic leachates from leachate exposure experiment that were inoculated with ciprofloxacin.

Treatment ID	Leachate concentration (mg/mL)
C3	0.0775
C5	0.0031
C8	0.0000248
C0	0

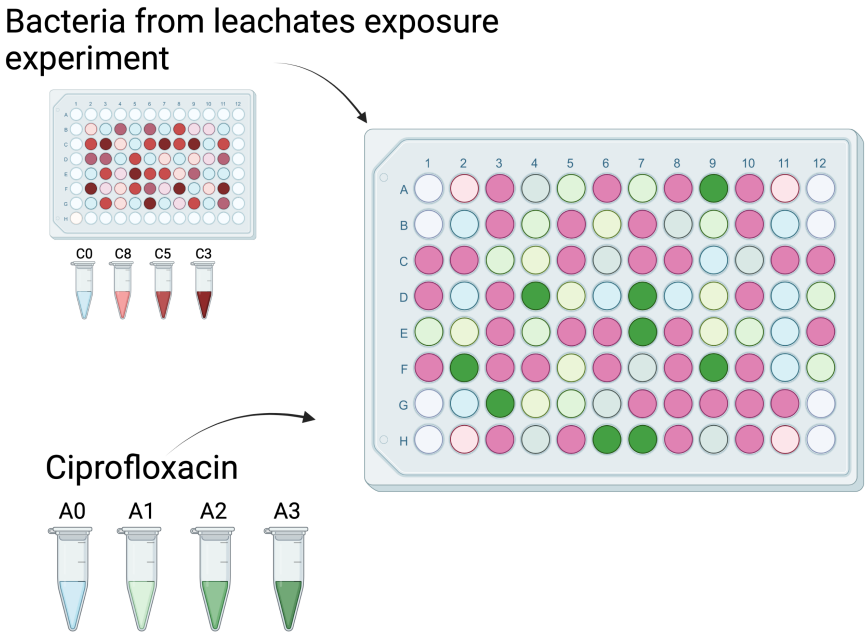

Figure 6. Illustration of the ciprofloxacin exposure experiment. Enrichments previously exposed to selected concentrations of leachates were inoculated with three different concentrations of ciprofloxacin in a 96 well plate. The bacterial growth was measured using optical density at 630 nm. The illustration was made using Biorender.com.

2.5 Growth curve parameter processing

Data processing of the optical density curves obtained from the absorbance measurements at 630 nm included subtracting the values from the blank samples from all data points to remove effects of medium. For the first experiment, the ten first measurements were subtracted from all samples to reduce the initial noisiness. Then the data was analysed using the "*growthrates*" package in R (Petzoldt, 2020), with functions based on methods from Hall et al. (2014) and

Kahm et al. (2010), to obtain growth curve parameters of interest: maximum growth rates, yield (maximum optical density) and duration of lag phases (figure 7). The package contains three methods to analyse the data, of which the two first were chosen in this project: 1) identifying the period of exponential growth, and fitting linear models to this period, 2) using smoothing splines methods to allow for non-parametric growth rate estimations and 3) fitting non-linear models to parametric growth models. For more information on this package, see (Petzoldt, 2020). Using this package, the lag phases were identified using linear model fitting of log transformed data, and two different methods were used to find the maximum growth rates; either linear model fitting on log transformed data or using non-parametric smoothing splines. Both were used to identify potential differences arising from the different methods, however in this project, the methods produced quite similar results. The yield was obtained by finding the mean of the 20 highest optical density measures of each bacterial community. For the leachates experiment, the first ten values were removed from the growth curves to prevent inaccurate fitting of the growth parameter models, as the absorbance measurements were highly noisy. However, in the ciprofloxacin exposure experiment, no values were subtracted, as exponential growth started almost immediately after the first measurement of absorbance in many incubations. In wells with no bacterial growth, the growth parameters were manually corrected after model fitting, i.e., lag phases were sat to 97.5 hours, the maximum growth rates (both linear and smoothed) and yield were set to 0, as these were inaccurately assigned due to high noise to response ratio in the control treatments by the "*growthrates"* package in R.

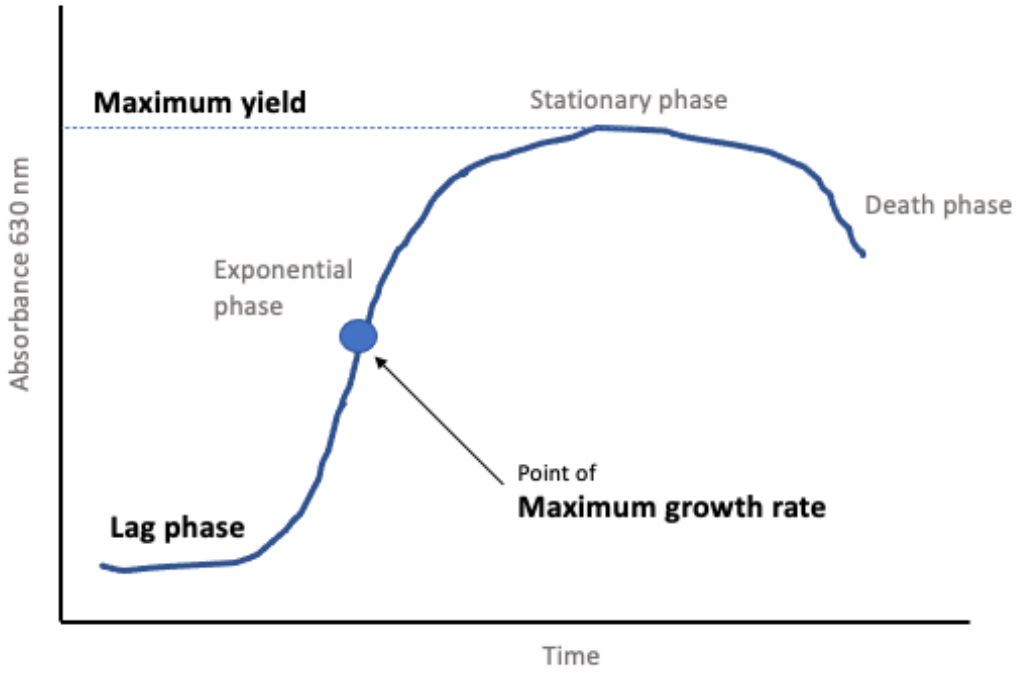

Figure 7. Bacterial growth curve. The relevant growth curve parameters for this project are highlighted in bold, and include the duration of the lag phase, the maximum growth rate, and the yield (maximum optical density).

2.6 DNA extraction

DNA metabarcoding allows for efficient biodiversity investigations (Taberlet et al., 2012) and is thus an appropriate tool for analysing bacterial biodiversity changes resulting from toxicant exposure (Hjelset, 2021; Yang et al., 2018; Zhang et al., 2018) such as leachates and ciprofloxacin. A metabarcoding workflow typically involves several steps: sampling, DNA extraction and amplification, DNA sequencing and bioinformatics (M. Liu et al., 2020). DNA extractions were performed in two different stages of the experiment: 1) the bacteria remaining after all samples reached stationary phase from the leachate exposure experiment and 2) the bacteria remaining after all samples had reached stationary phase after the ciprofloxacin exposure experiment. The bacterial DNA from the exposure experiments were extracted using Qiagens DNeasy Blood & Tissue kit, with the Pretreatment for Gram-Positive bacteria. DNA quantification of the DNA extracts was performed using Quant-it™ PicoGreen® assay with an inhouse protocol (Eiler, 2021) and a microplate reader (Synergy™ MX).

2.7 Amplicon library preparations

The gene regions of interest were the variable regions V3 and V4 of the bacterial 16s rRNA gene sequence. To amplify the bacterial gene sequences of interest, the illumina MiSeq Dual Index Amplicon Sequence Sample Preparation Bacterial 16S rRNA gene protocol (Juottonen et al., 2020) was used. The bacterial primers 341F (Herlemann et al., 2011) and 805RN (Apprill et al., 2015) were used for amplification. The primer sequences were:

> Illumina adapter-N4-341F: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCCTACGGGNGGCWGCAG-3'
> Illumina adapter-805NR: 5'-AGACGTGTGCTCTTCCGATCTGACTACNVGGGTATCTAATCC-3'

The PCR reactions were performed in a Mastercycle ep gradient S (Eppendorf) following instructions in the PCR protocol. However, the number of cycles was adjusted to 35 in the first PCR reaction, as no amplification was observed in the original setting. The PCR products were assessed using gel electrophoresis to ensure successful amplification. This was followed by purification of the PCR products using Agencourt AMPure XP purification protocol, involving magnetic AMpure XP beads. PCR reaction products were quantified using Quant-it™ PicoGreen® assay (Eiler, 2021) and a microplate reader (Synergy™ MX). The samples were pooled and submitted to the Norwegian Sequencing Center (NSC, Department of Medical Genetics, Ullevål, Oslo, Norway), where sequencing was conducted with MiSeq v3 600 cycles and 10% PhiX to obtain paired end reads.

2.8 16S rRNA gene amplicon processing

2.8.1 Raw sequence processing and dada2 pipeline

Demultiplexed sequences were received from NSC, upon which cutadapt (V.1.18) was used to remove primers and adapters. The R package "*Dada2"* (Callahan et al., 2016) was used to further process the reads. This process involved dereplication, filtering and trimming, sample inference, merging of paired reads, removing chimaeras and assigning taxonomy using the Silva 138 database (Yilmaz et al., 2014). Inspection of the error plots produced from the dada2 pipeline identified relatively poor sequence results, thus two methods were used to overcome this challenge: 1) the quality phred score was set to 5 and 2) the quality phred score at 20. In the latter case the reads were concatenated when merging the filtered and trimmed reads. The dada2 pipeline is quite robust to reads of lower quality, as it incorporates the quality profile in the error model (Callahan et al., 2016), therefore utilisations of a quality phread score of 5 was used. The results of both reads processing methods were highly comparable, and the first processing method was chosen, since it resulted in a much higher number of sequences, necessary for alpha and beta diversity estimations. During filtering and trimming, the forward and reverse reads were truncated at 245 and 215 base pairs for the first method respectively, and for the second method, the forward and reverse reads were truncated at 200 and 160 base pairs respectively, based on the phred score plots.

2.8.2 Analysis of sequences after dada2

To further assess the sequences, the R package "*phyloseq*" (McMurdie & Holmes, 2013) was used to align sequence data with metadata and construct a phyloseq object. Some contamination occurred, resulting in reads found in the negative controls. The contamination was assessed by removing all sequences found in the negative samples from all samples. This was done using the R package "*decontam*" (Davis et al., 2017). After decontamination, non-bacterial sequences were removed, including sequences originating from mitochondria and chloroplasts. Rarefaction was performed to obtain an equal amount of reads in each sample (equal sequencing depth), making it possible to compare alpha diversity estimates between samples without introducing biases from different sample sizes (Willis, 2019). Rarefaction in this experiment was based on the sample with minimum number of reads ("C2-01" from leachate experiment and "C3A3-03" from ciprofloxacin experiment). Alpha diversity measures included abundance-based coverage estimator (ACE) (for detailed information on ACE method, see (Chao & Lee, 1992), and Simpson's index (for detailed information on the Simpson index see (Simpson, 1949). The ACE richness index, starting at one, indicating one species being present, is a measure of the richness of diversity in the samples, i.e. the number of ASVs present in the samples. The higher the ACE richness index, the greater the diversity. The Simpson index is a measure of the diversity ranging from 0-1, taking both the number of ASVs present and their relative abundance into account. The Simpson index is often described

as the probability of choosing the same species in a community twice (Somerfield et al., 2008). As such, a community with high Simpson index is commonly considered to be of low abundance, where 1 represents no diversity, and thus a low Simpson index is a mark of a highly diverse community, with 0 representing infinite diversity.

The beta diversity is a measure of the difference in diversity between two communities, or in this project: treatments. We used Bray-Curtis distances to assess beta diversity, which is a commonly used beta-diversity estimator, see e.g Gardham et al. (2014), Gillmore et al. (2021) and Meredith et al. (2021). Bray-Curtis distance estimates differences in abundance between two communities (for detailed information on the formula behind, see (Bray & Curtis, 1957)).

2.9 Statistical analysis

2.9.1 Growth curve parameters and alpha diversity estimates

The experiments conducted in this project were factorial and balanced, meaning that all bacterial enrichments received all treatments randomly. This was done to minimise possible confounding effects. Statistical analysis was performed in RStudio version 1.2.1335. Data was fitted with linear modelling, and plots of the fitted response variables were visually inspected to assess whether assumptions were met. Normality of residuals and homoscedasticity assumptions in regression analysis were further assessed using Shapiro Wilks-test ($p.significant < 0.05$) and the Goldfeld-Quandt test (Goldfeld & Quandt, 1965) ($p.significant < 0.05$) respectively. For data not meeting assumptions of linearity and homoscedasticity, the "*fitdistrplus*" package (Delignette-Muller & Dutang, 2015) in R was used to identify the best distribution. A model was fitted with the chosen distribution, and by visual inspection of the fitted plots, the best distribution was chosen. To obtain models we used generalised linear modelling – glm function in R, with identity-linked gamma distribution. This model was chosen because it allows for heteroscedasticity and estimates relationships with the y-axis where the intercept does not contain zero (Larsen et al., 2011). The glm-models were checked for overdispersion by dividing the residual deviance on the residual degrees of freedom. If the result is below 1, then there is no overdispersion and the variance in the data set is not larger than the model assumption's variance. See Cox (1983) for more remarks on overdispersion. Only models not showing overdispersion were used. Standardised parameters for both linear and general linear regression, allows comparing the effects of the independent variables against each other, and was obtained by using a standardised version of the dataset in a model. In cases where the distribution was closest to a normal distribution, although not meeting the normal assumptions, but the assumptions of heteroscedasticity were met, linear models were fitted, as the normality-assumption being violated may still produce reliable estimations (Knief & Forstmeier, 2021). To further confirm that the model was appropriate, comparisons between these non-normal linear models and generalised linear models showed

that the linear models produced predictions most in line with the observed dataset. The Wald approximation was used to obtain confidence intervals and p-values.

The controls were assessed using ANOVA (a specialised variant of linear model), Kruskal Wallis or PERMANOVA to check: 1) whether there was a difference between the samples treated only with DMSO and bacteria with no treatments. This was done to ensure that the effects observed from leachates were in fact from the leachates and not the DMSO used in the leachate preparations, and 2) that the bacteria did in fact respond to the treatment, using a positive control of 3,5 DCP for the leachate experiment and high concentration of ciprofloxacin on the ciprofloxacin experiment. Assumptions for ANOVA were checked as described for linear regression models, and homogeneity in variance was checked using Levene's test (p.significant < 0.05).

2.9.2 Beta diversity

Beta diversity was assessed using Bray-Curtis dissimilarity matrix, produced by standardising rarefied reads with Wisconsin scaling. A non-metric-multi-dimensional-scaling (NMDS) plot was made from the scores for visual analyses. Permutational multivariate analysis of variance (PERMANOVA) was used on the Bray-Curtis distance matrix to identify any possible differences produced by leachates or ciprofloxacin and leachates on centroids of the microbial community structures. Where differences were observed, a pairwise PERMANOVA was applied (using "*pairwise.adonis*" function in R (Martinez Arbizu, 2020)). To ensure equal point dispersion, a permutest was performed on all PERMANOVA models. To further understand effects on beta diversity, heatmaps were made using Euclidean distances for visual inspections of clustering of treatments, using the "*pheatmap*" package (Kolde, 2019) in R.

To investigate changes in individual bacterial taxa, Deseq2 package in R (Love et al., 2014) was used. This package uses empirical Bayes techniques for its shrinkage estimations of log fold changes, which allows for testing whether individual bacterial genera were affected by the treatment, even in cases of relatively low sample size (Love et al., 2014). Deseq2 uses Wald test for significance, with adjustments for multiple testing using Benjamin and Hochbergs procedure (Love et al., 2014). As this method uses a rlog transformation (Love et al., 2014), non-rarefied reads are used as input for the model.

2.10 Visualisation of workflow

See figure 8 for a visualisation of the main elements of the project.

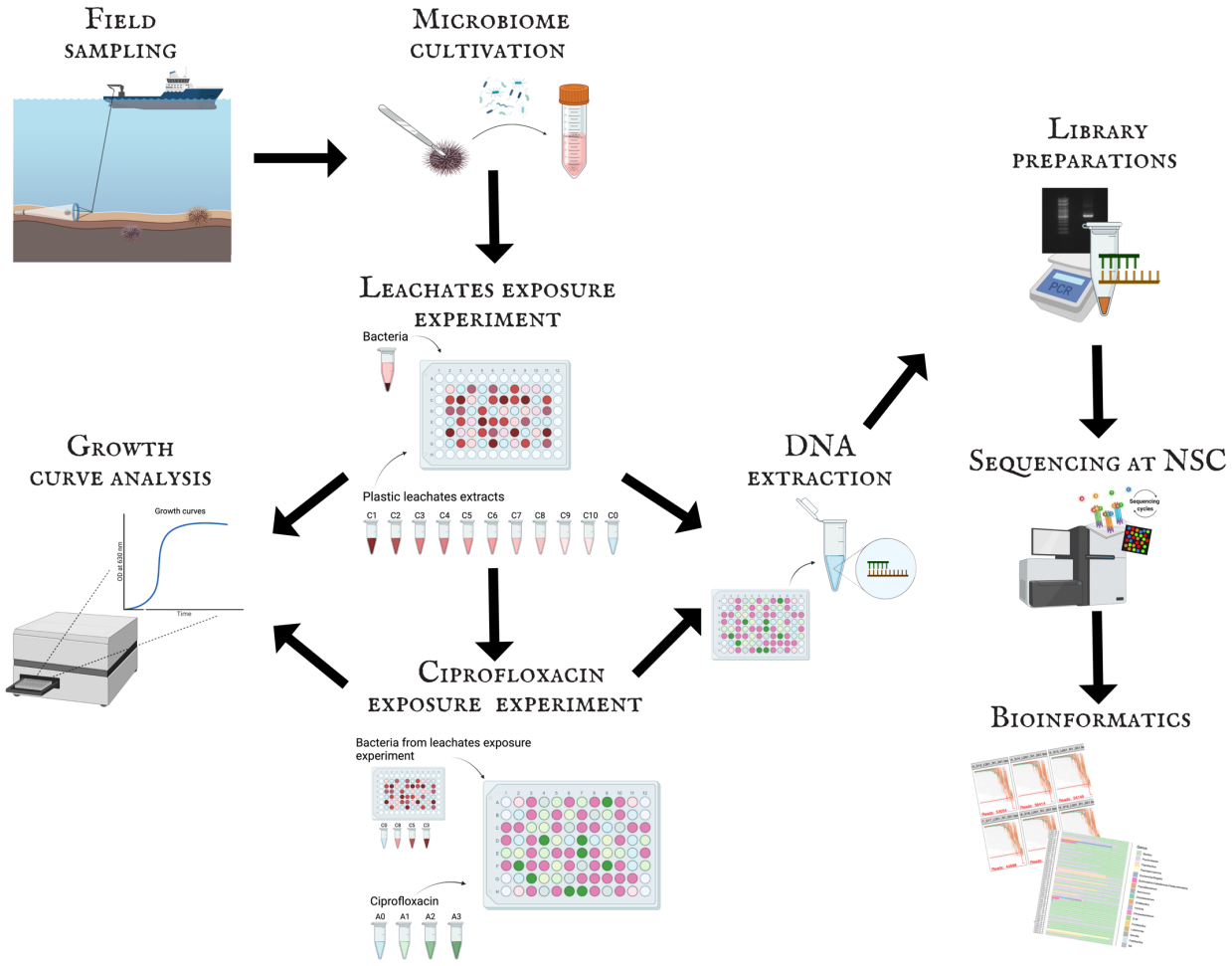

Figure 8. Visualisation of the workflow for the master project. The illustration was created using biorender.com and canva.com.

3 Results

3.1 The effects of leachates on bacterial communities

3.1.1 Bacterial community growth

The growth curves were based upon observations in 60 wells (including controls and blanks) with four replicates per treatment. The bacterial communities were variable among replicates, however, there were trends in response to the different treatments (figure 9).

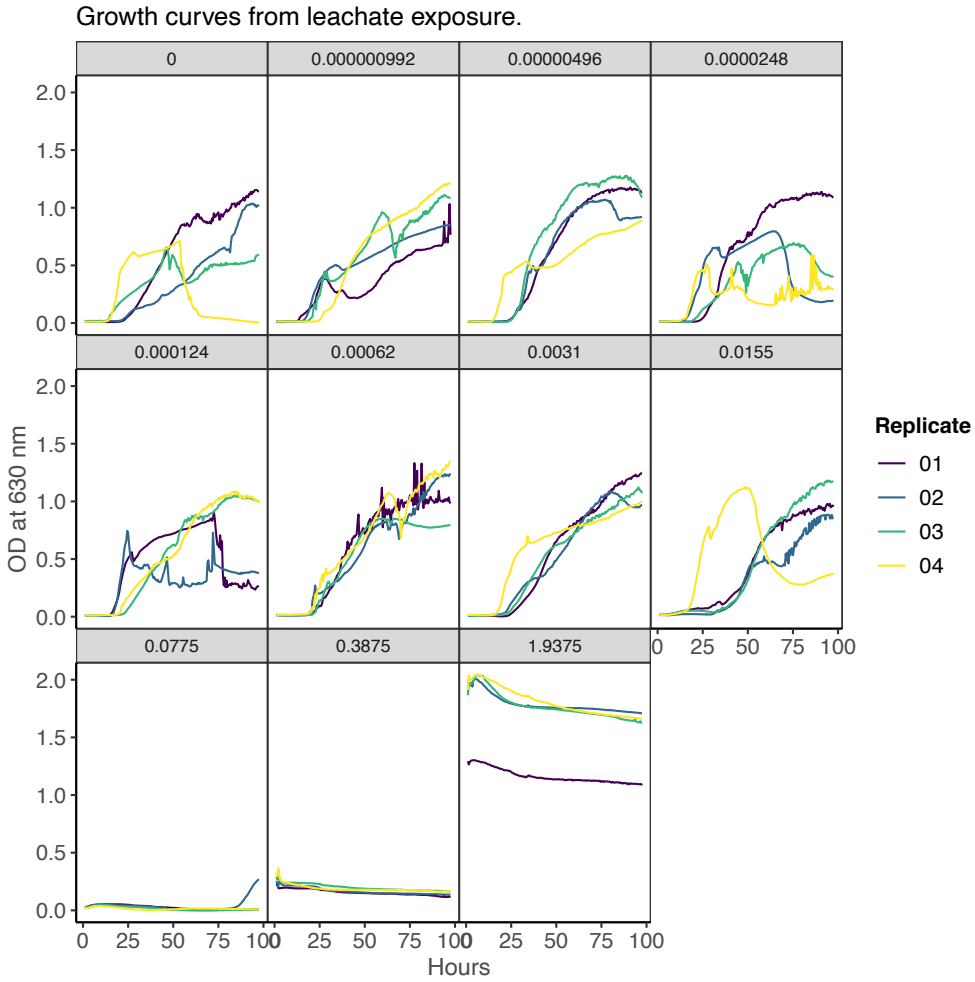

Figure 9. Growth curves obtained using optical density measurements at 630 nm from the leachate experiment. Each box represents enrichments exposed to the specific leachate concentrations (mg/mL), as indicated above the boxes. The four different lines illustrate each of the four replicates per enrichment.

The maximum yield ranged from 0 OD in the communities with no growth, and up to 1.27 OD in the untreated bacterial community. The linear maximum growth rates ranged from 0 to

0.37 OD change per hour (OD/h), while the smoothed maximum growth rates ranged from 0 to 0.26 OD/h. The bacterial enrichments exposed to the highest concentrations of leachates had no observable growth, while highest maximum growth rates were found in enrichments with the four lowest concentrations of leachates or no leachate exposure. The shortest lag phase lasted for 13.3 hours and occurred in the treatments with no leachate exposure. The longest lag phases (97.5 hours) were observed in bacteria treated with the three highest concentrations of leachates representing incubations with no observable growth. See figure 10 for responses to the treatments in each growth parameter.

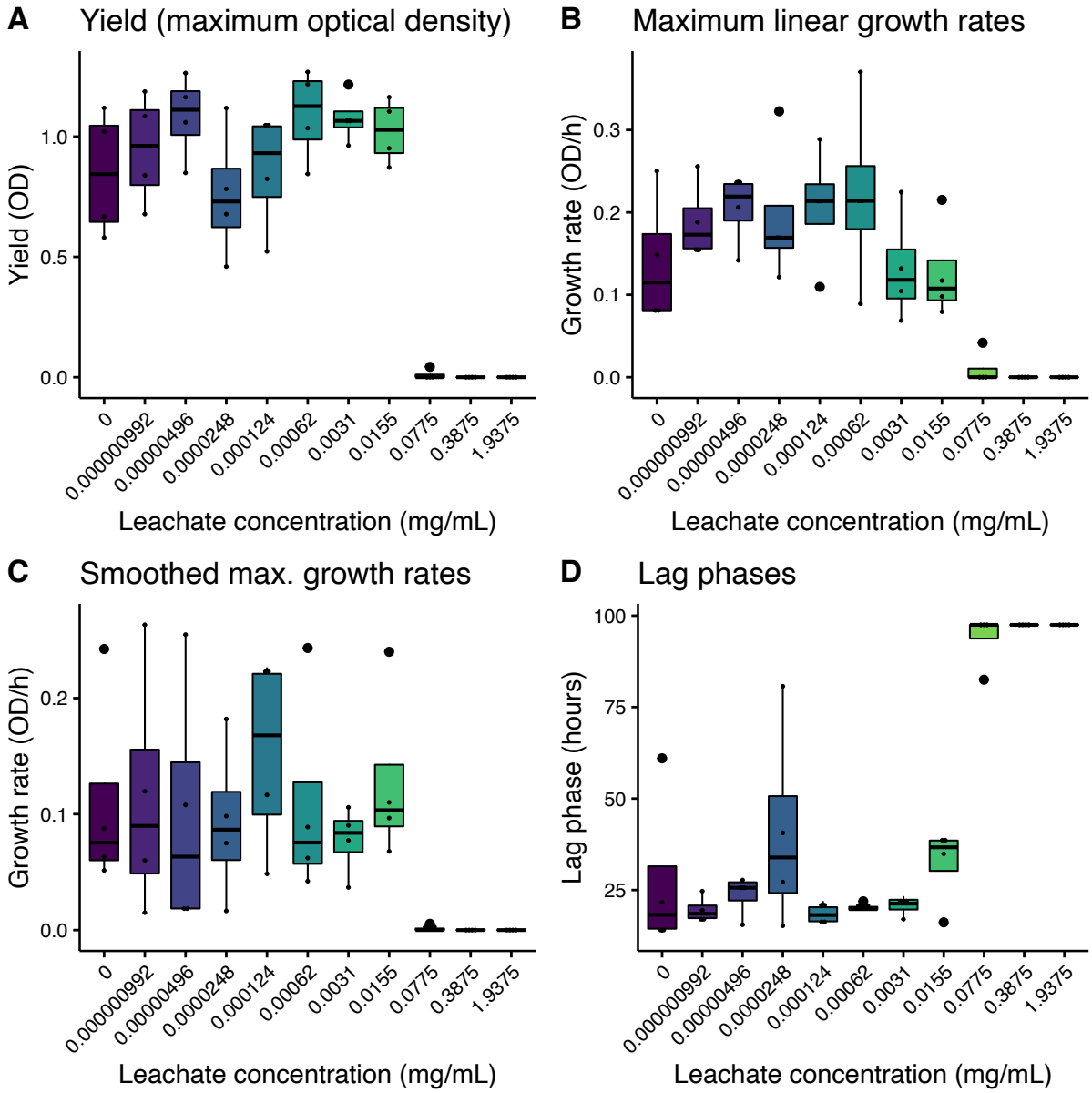

Figure 10. Boxplots of the observed growth curve parameters. The dots represent each observation, with a total of four observations per treatment. The boxes represent the first and third quantiles, while the minimum and maximum values are represented by the whiskers. The thick, horizontal lines represent the median. Plot A illustrates the yield (maximum optical density) from each incubation. Plot B illustrates the maximum linear growth rates for each incubation. Plot C illustrates the maximum smoothed growth rates for each incubation, and plot D illustrates the lag phases from each incubation.

Linear models were fitted to predict effects of leachates on growth parameters including yield and maximum growths as estimated by linear and splines models, while a glm-model with gamma distribution and identity-link was fitted to predict effects on lag phase (figure 11). For all growth parameters, negative effects of leachates (or increase in lag phase) started to be observable at concentrations around 0.1 mg/mL (visual inspection of predicted growth parameters). For yield (maximum optical density), the variance in the model captured a significant and substantial proportion of the variation in the data ($R^2 = 0.35$, $F(1, 42) = 22.46$, $p < 0.001$, adj. $R^2 = 0.33$). The intercept which corresponds to leachate concentration of 0 mg/mL was at 0.81 OD (95% confidence interval (CI) [0.68, 0.94], $t(42) = 12.97$, $p < 0.001$). The effect of leachates was negative and significant (beta = -0.50, 95% CI [-0.71, -0.28], $t(42) = -4.74$, $p < 0.001$; Std. beta = -0.59, 95% CI [-0.84, -0.34]).

In the case of the growth rates estimated by linear models a significant and moderate proportion of the variation was captured by leachate concentrations ($R^2 = 0.15$, $F(1, 42) = 7.30$, $p = 0.010$, adj. $R^2 = 0.13$). The intercept, representing the bacteria not treated with leachates, was at 0.09 OD/h (95% CI [0.07, 0.12], $t(42) = 7.40$, $p < 0.001$). A significant and negative effect of leachates on growth rates was found (beta = -0.06, 95% CI [-0.10, -0.01], $t(42) = -2.70$, $p = 0.010$; Std. beta = -0.38, 95% CI [-0.67, -0.10]), meaning that maximum growth rates decreased with increasing leachate concentrations.

The proportion of variance explained by the model for maximum growth rates estimated by splines models (smoothed maximum growth rate) was substantial and significant ($R^2 = 0.26$, $F(1, 42) = 14.78$, $p < .001$, adj. $R^2 = 0.24$). The intercept of the model corresponds to leachate concentration of 0 mg/mL, and was at 0.15 OD/h (95% CI [0.12, 0.18], $t(42) = 10.51$, $p < 0.001$). The effects of leachates on maximum linear growth rates were negative and significant (beta = -0.09, 95% CI [-0.14, -0.04], $t(42) = -3.84$, $p < 0.001$; Std. beta = -0.51, 95% CI [-0.78, -0.24]), meaning that maximum growth rates decreased with increasing leachate concentrations.

In the case of lag phases, leachate concentrations could explain a substantial proportion of the observed variation with an intercept (representing no exposure) at 29.61 hours (95% CI [22.94, 38.59], $t(42) = 8.80$, $p < 0.001$, Nagelkerke's $R^2 = 0.47$). Leachates induced a positive and significant effect on lag phase duration (beta = 110.66, 95% CI [38.99, 260.38], $t(42) = 3.24$, $p = 0.001$; Std. beta = 62.03, 95% CI [21.85, 145.94]) , meaning that lag phase increased with increasing leachate concentrations.

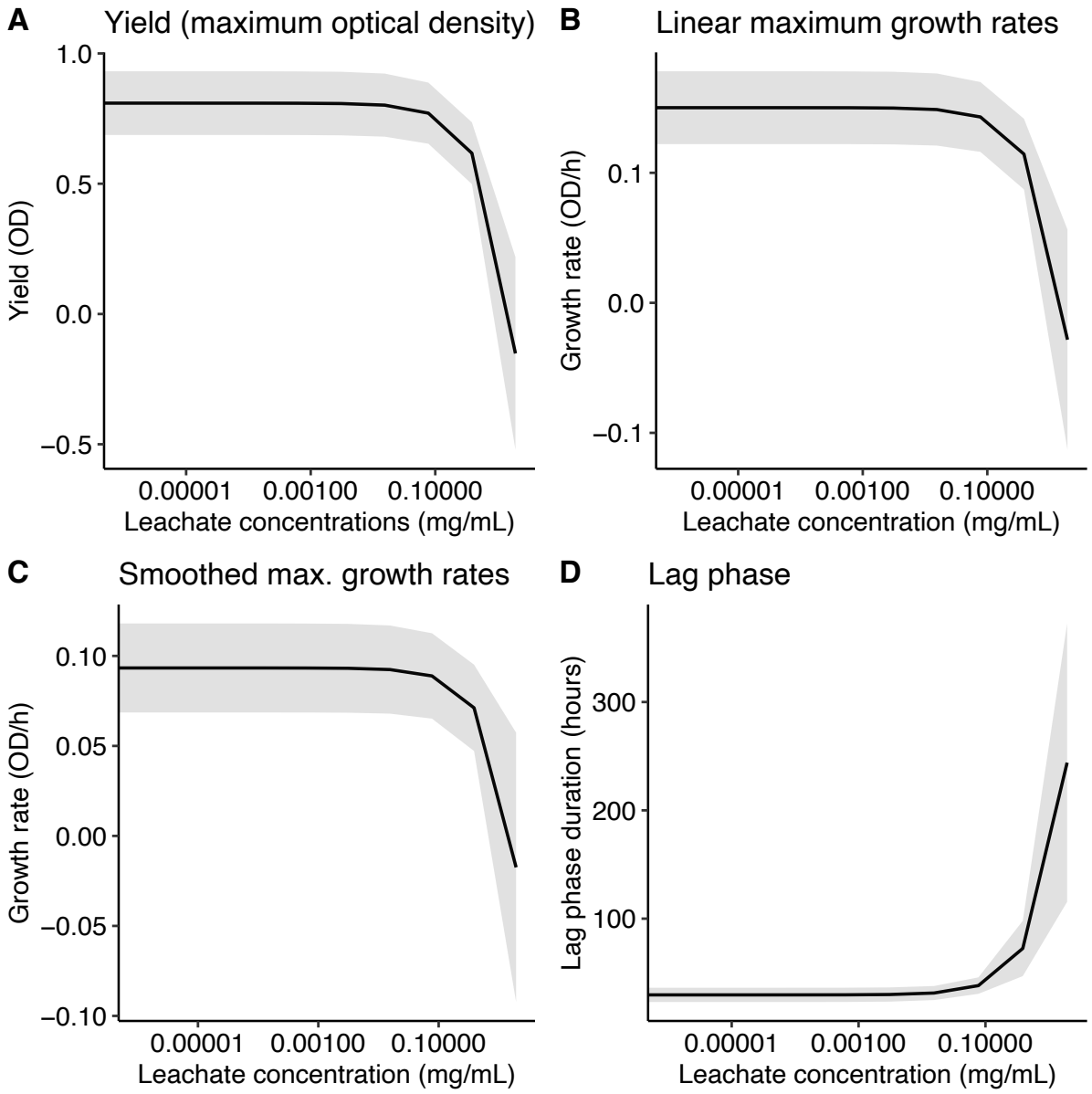

Figure 11. Predicted growth curve parameter- regressions in response to leachate concentrations (mg/mL) from the leachate experiment. The light grey colour around the regression line indicates the corresponding confidence interval. Plot A illustrates the yield (maximum optical density) from each incubation. Plot B illustrates the maximum linear growth rates for each incubation. Plot C illustrates the maximum smoothed growth rates for each incubation, and plot D illustrates the lag phases from each incubation.

In yield, the positive control was significantly different from bacteria in the untreated incubations, with a large effect size (F(1, 6) = 19.10, p = 0.005; Eta2 = 0.76, 95% CI [0.31, 1.00]). In both maximum growth rates estimates and lag phases, the differences were not significant (linear: F(1, 6) = 0.77, p = 0.415; Eta2 = 0.11, 95% CI [0.00, 1.00], smoothed: F(1, 6) = 0.15, p = 0.715; Eta2 = 0.02, 95% CI [0.00, 1.00] and lag phase: Kruskal-Wallis chi-squared = 3, df = 1, p-value = 0.08 respectively). No significant differences were observed between untreated bacteria and bacteria exposed to DMSO in any of the growth parameters (maximum yield (F(1, 6) = 0.66, p = 0.446; Eta2 = 0.10, 95% CI [0.00, 1.00]), linear maximum growth rate (F(1, 6) = 0.62, p = 0.463; Eta2 = 0.09, 95% CI [0.00, 1.00]), smoothed maximum growth rate (F(1, 6) =

0.34, p = 0.580; Eta2 = 0.05, 95% CI [0.00, 1.00]) and lag phase (Kruskal-Wallis chi-squared = 0.08, df = 1, p-value = 0.77), indicating that effects observed are in fact induced by leachates.

3.2 Bacterial community identification

From 160 libraries that were submitted to the Norwegian Sequencing Centre (UiO), a total of 8 319 442 raw sequences were returned. After filtering, denoising and removing chimaeras, a total of 3 788 028 reads were distributed among 159 samples. After removing reads found in negative controls using "*decontam"* package in R (Davis et al., 2017), and removing reads from chloroplast and mitochondria, a total of 3 302 099 reads were distributed among 139 samples, with a mean of 23756 reads, ranging from 2058 to 55845 reads per sample. Rarefaction was done individually on each dataset from the two exposure experiments, based on the minimum number of reads from each experiment (4978 (figure 12) and 2058 (figure 13) for leachate experiment and ciprofloxacin experiment respectively. After rarefaction, 280 out of 284 taxa from 55 samples remained in the leachate experiment, and 212 out of 231 taxa from 84 samples in the ciprofloxacin experiment.

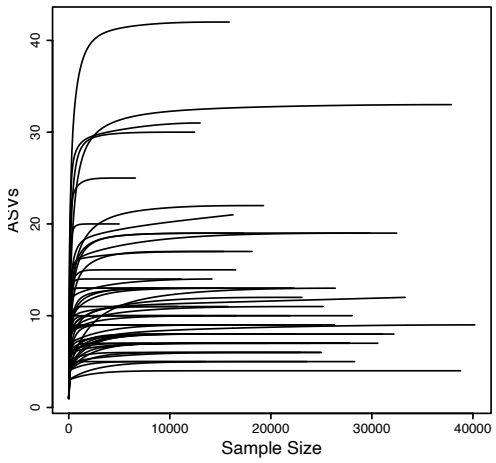

Figure 12. Rarefaction curve of the ASVs from the leachate experiment.

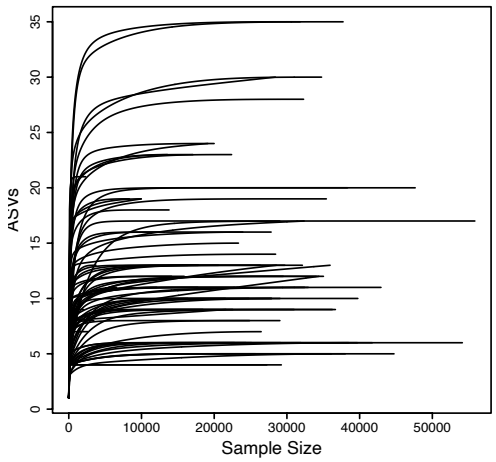

Figure 13. Rarefaction curve of the ASVs from the ciprofloxacin experiment.

3.3 Bacterial community composition responses to leachates

The most abundant genera among all samples in the leachate exposure experiment were *Bacillus*, *Psychrobacter*, *Paenibacillus*, *Lysinibacillus*, *Sporosarcina* and *Bacteroides*. See figure 14 for an overview of bacterial genera distributed over different samples and the proportion of reads in each sample.

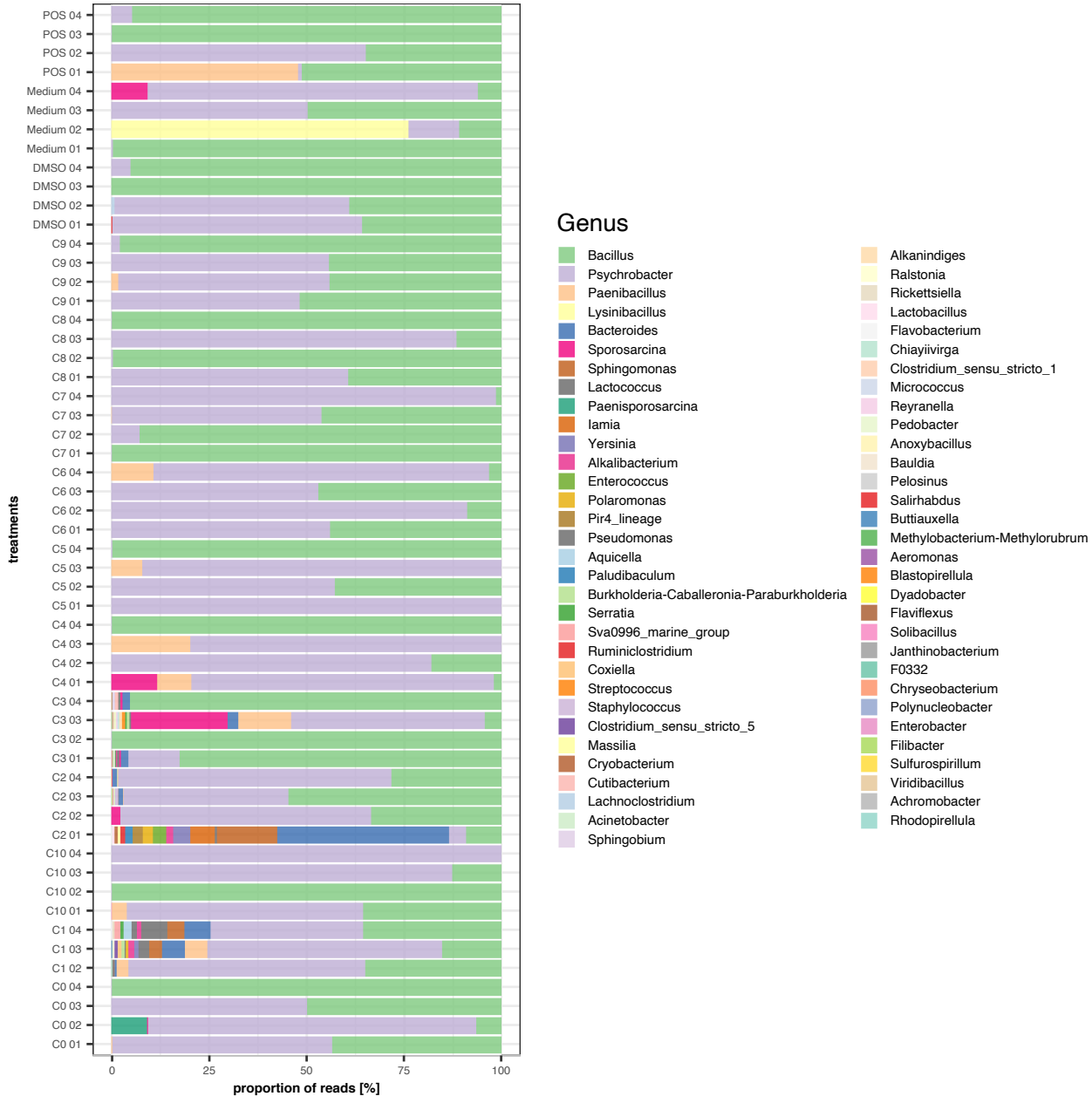

Figure 14. The proportion of reads of each bacterial genera in the different leachate treatments. Each colour represents an individual genus. This plot was based on rarefied datasets. The samples named "C.." indicate the concentration of leachates, with C1 representing the highest leachate concentration and "C10" representing the lowest concentration of leachates. "DMSO" are the samples exposed to DMSO only, "POS" are the positive controls, and "Medium" are samples with only YCFA medium and bacteria. The number to the left (01-04) indicates the replicate number.

For bacteria exposed to leachate concentrations in the range 0-15.5 μg/mL, ACE richness was between 1-20. At concentrations above this, there was an increase in ACE richness index up to 40. A similar pattern is not evident in the Simpson index, where the estimates ranged between 0-0.8, although there seemed to be an increase in the highest leachate concentrations. See figure 15 for boxplots of the observed alpha diversity indexes.

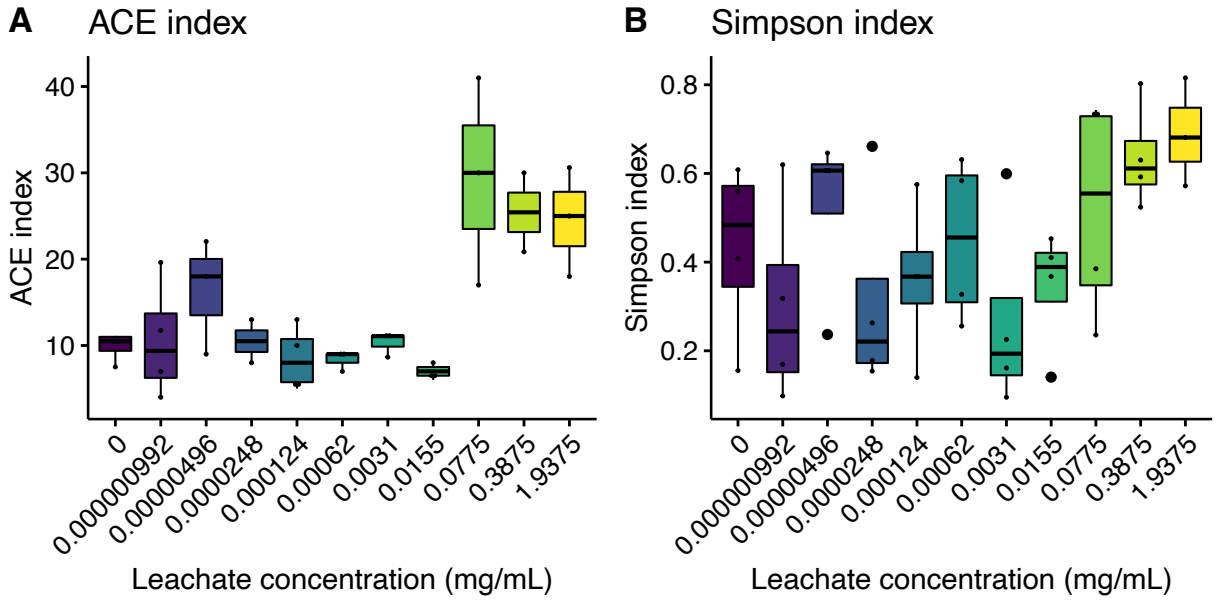

Figure 15. Boxplots of the observed alpha diversity metrics resulting from the leachate exposure experiment. The dots represent each observation, with a total of four observations per treatment. The boxes represent the first and third quantiles, while the minimum and maximum values are represented by the whiskers. The thick, horizontal lines represent the median. Plot A illustrates the ACE index estimates in response to leachate concentrations (mg/mL), while plot B illustrates the estimated Simpson indexes in response to leachates concentrations (mg/mL).

Linear models, explaining significant, but moderate proportions of the variance, was fitted to assess effects of leachates on ACE richness (R2 = 0.20, F(1, 33) = 8.43, p = 0.007, adj. R2 = 0.18) and Simpson indexes (R2 = 0.16, F(1, 41) = 7.91, p = 0.008, adj. R2 = 0.14). These models' intercepts, corresponding to no leachate exposure, were at 12.37 (95% CI [9.41, 15.33], t(33) = 8.50, p < 0.001) for ACE richness and at 0.40 (95% CI [0.34, 0.47], t(41) = 12.55, p < 0.001) for Simpson index. Significant and positive effects of leachates were identified on both ACE richness (beta = 7.35, 95% CI [2.20, 12.50], t(33) = 2.90, p = 0.007; Std. beta = 0.45, 95% CI [0.13, 0.77]) and Simpson index (beta = 0.17, 95% CI [0.05, 0.30], t(41) = 2.81, p = 0.008; Std. beta = 0.40, 95% CI [0.11, 0.69]) (figure 16).

To ensure that effects observed did not come from DMSO, but rather leachates, control samples with bacteria enriched in medium were compared against samples exposed to DMSO. No significant differences in any of the alpha diversity measures was found between these controls (ACE: F(1, 5) = 0.34, p = 0.586; Eta2 = 0.06, 95% CI [0.00, 1.00] and Simpson: F(1, 6) = 0.05, p = 0.833; Eta2 = 8.02e-03, 95% CI [0.00, 1.00]). The positive controls consisting of bacteria exposed to 3,5 DCP were not significantly different in either ACE richness nor Simpson

index compared to the unexposed bacteria (ACE: F(1, 4) = 0.49, p = 0.522; Eta2 = 0.11, 95% CI [0.00, 1.00] and Simpson: F(1, 6) = 1.24e-05, p = 0.997; Eta2 = 2.07e-06, 95% CI [0.00, 1.00]).

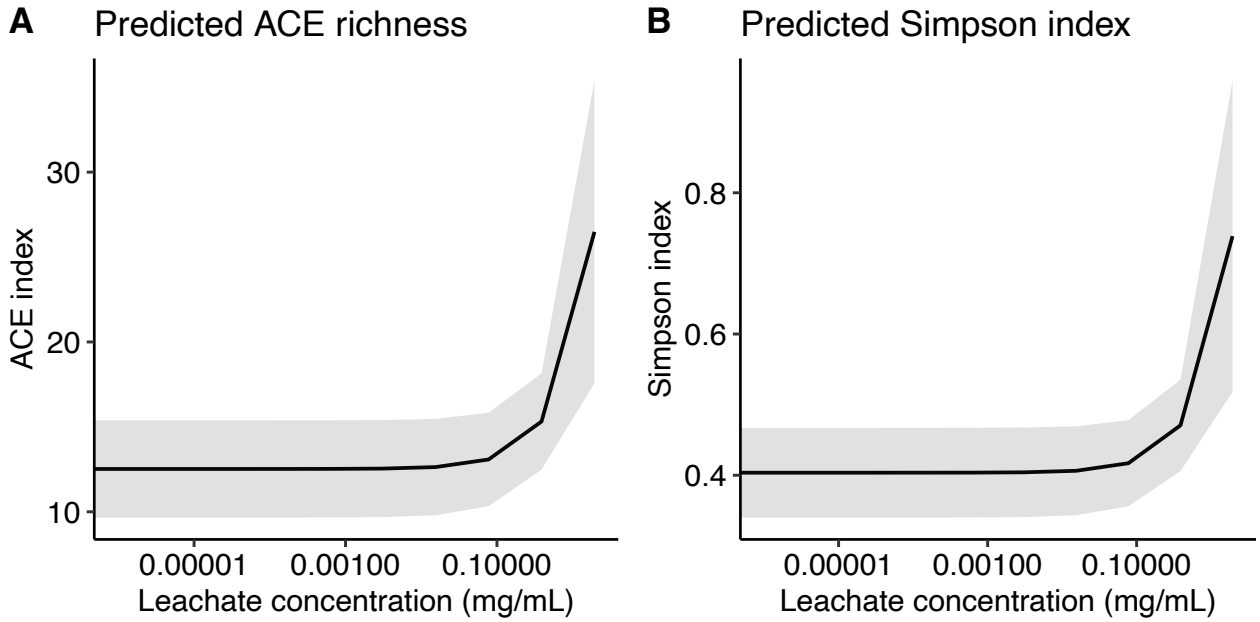

Figure 16. Predicted alpha diversity metric-regressions obtained from linear modelling in the leachate experiment. The light grey colour around the regression line indicates the corresponding confidence interval. Plot A illustrates predicted ACE richness estimates. Plot B illustrates predicted Simpson index estimates.

Beta diversity was assessed for 51 samples in the leachate experiment. Visualisation in non-metric Multidimensional Scaling (NMDS) plot (figure 17) revealed no clear effects of leachates on Bray-Curtis dissimilarity distance. Assessing Euclidean distances using a heatmap further confirmed no clustering based on leachate concentrations (figure 18). This was confirmed by PERMANOVA (F(10) = 0.26, p = 0.156, R2 = 0.26, permutations = 999). No significant differences between the various controls were observed (PERMANOVA, F(1) = 1.11, P-value =0.301, R2 = 0.07).

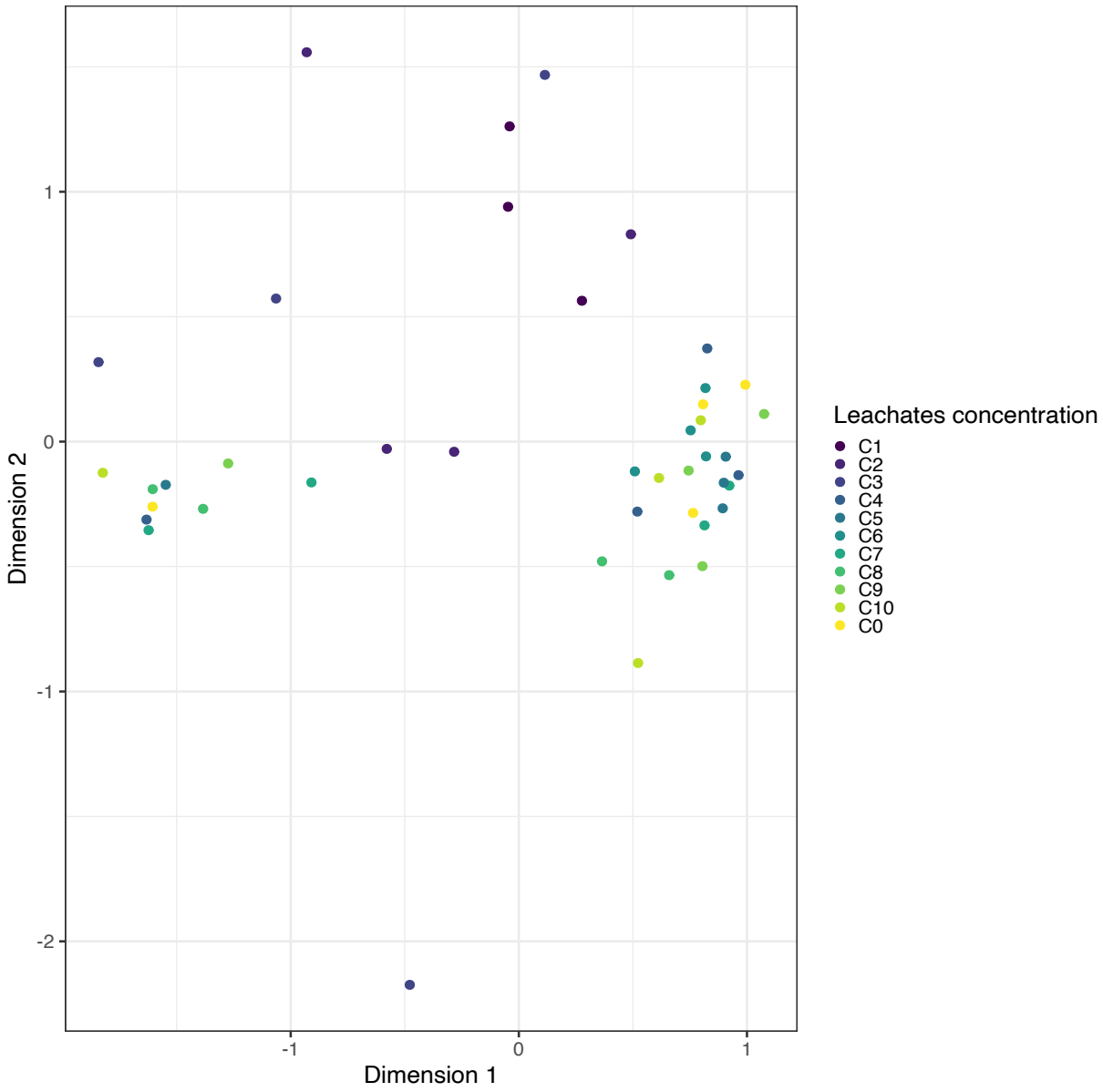

Figure 17. Beta-diversity in response to leachate exposure visualised in a non-metric Multidimensional Scaling (NMDS) plot. Samples that cluster together indicate high similarity. The samples named "C.." indicate the concentration of leachates, with "C1" representing the highest leachate concentration and "C10" representing the lowest concentration of leachates, and "C0" indicating no leachate exposure.

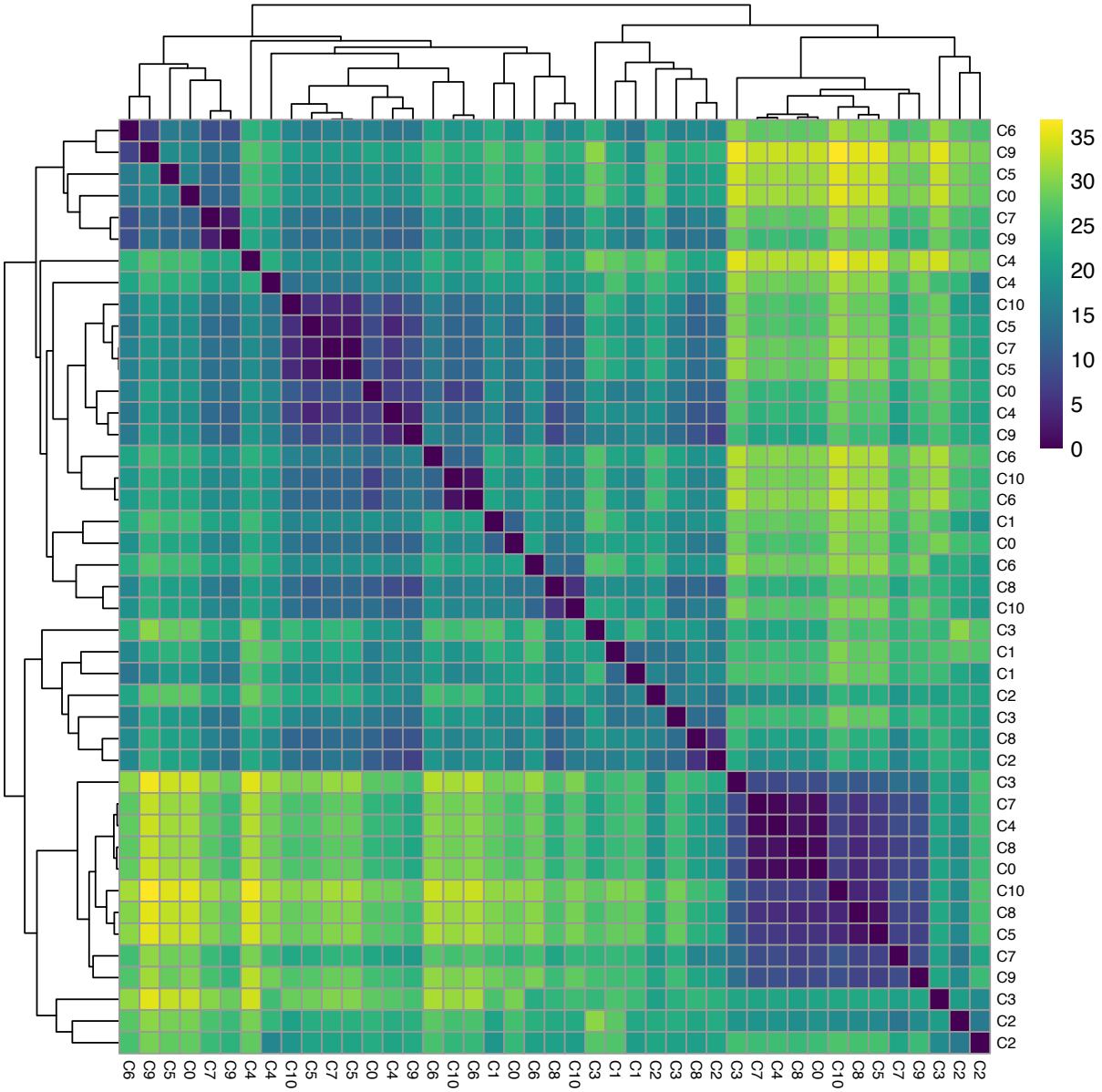

Figure 18. Beta diversity of the leachates exposed enrichments, illustrated using Euclidean distances to visualise differences from treatments in the enrichments. The colour gradient illustrates the differences: squares coloured yellow represents the most distant samples, while the purple colour represents the most similar samples. The clustering of the samples is illustrated at the top and to the left. The samples named "C.." indicate the concentration of leachates, with "C1" representing the highest leachate concentration and "C10" representing the lowest concentration of leachates, and "C0" indicating no leachate exposure.

To identify changes in individual amplicon sequence variants (ASVs) across the different treatments, a differential abundance analysis was performed using Deseq2. Comparisons were made between all leachate concentrations with the reference level set to unexposed enrichments. Eight bacterial ASVs had observed log fold changes, belonging to the genera *Bacillus*, *Paenibacillus*, *Psychrobacter*, *Sphingomonas*, *Lactococcus*, *Alkalibacterium* and *Sporosarcina*. Responses of ASVs belonging to *Bacillus* were highly variable, with both positive and negative trends to various levels of leachate exposure (figures 19 and 20). These changes

were often erratic changes since already at the lowest concentration of leachates both positive and negative significant log fold changes occurred. ASVs belonging to *Sphingomonas* had negative log fold changes under the two highest exposures. ASVs belonging to *Lactococcus* and *Alkalibacterium* had negative log fold changes in the three highest concentrations. *Psychrobacter* ASVs (figure 21) had significant positive log fold changes in response to treatment "C3", while *Sporosarcina* ASVs had negative log fold responses to treatments "C2" and "C3". *Paenibacillus* ASVs (figure 22) had negative log fold changes in treatments "C1", "C3" and "C4".

When comparing DMSO-exposed bacteria and the positive control against unexposed bacteria only two ASVs, belonging to the genera *Psychrobacter*, had significant negative log fold changes in the positive control compared to the unexposed bacteria (see the appendix for all plots showing significant log fold changes in the different treatments).

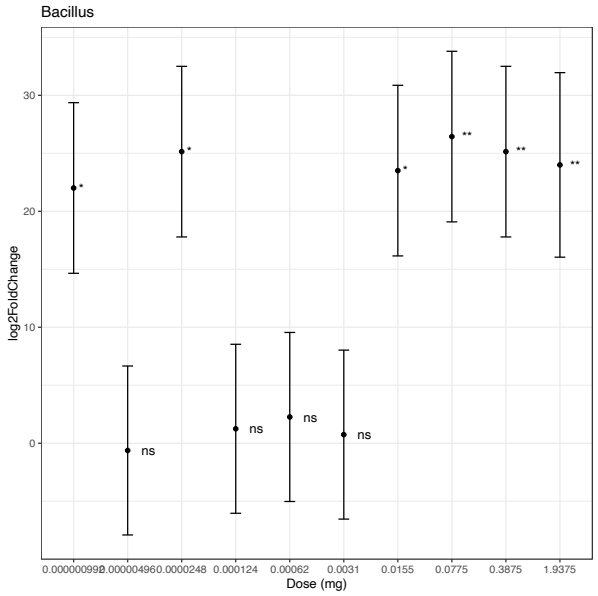

Figure 19. Log fold changes (y-axis) in the different concentration of leachates (x-axis) compared to the non-exposed enrichments. "ns" represent the non-significant changes, "" and "**" represents significant changes ($p<0.05$ and $p<0.01$ respectively).*

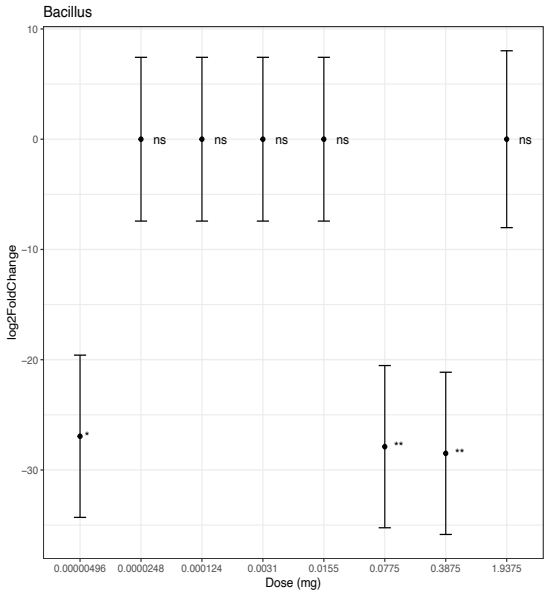

Figure 20. Log fold changes (y-axis) in the different tested concentration of leachates (x-axis) compared to the non-exposed enrichments. "ns" represent the non-significant changes, "" and "**" represents significant changes (p<0.05 and p<0.01 respectively).*

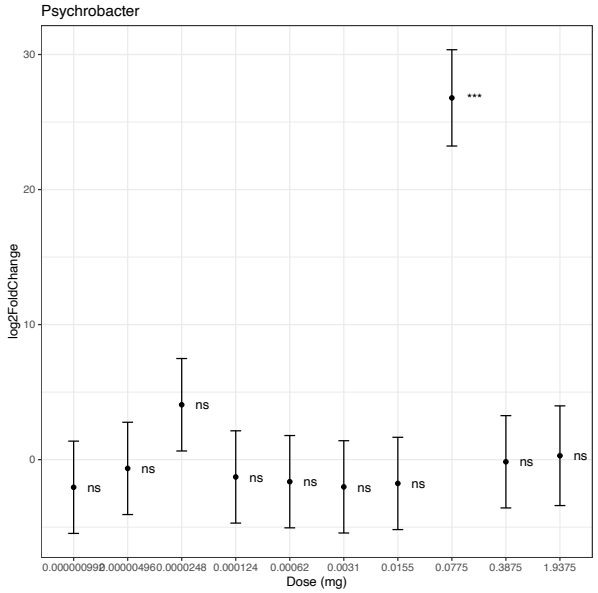

*Figure 21. Log fold change (y-axis) in the different concentration of leachates (x-axis) compared to the non-exposed enrichments. "ns" represent the non-significant changes and "***" represents significant changes (p<0.001).*

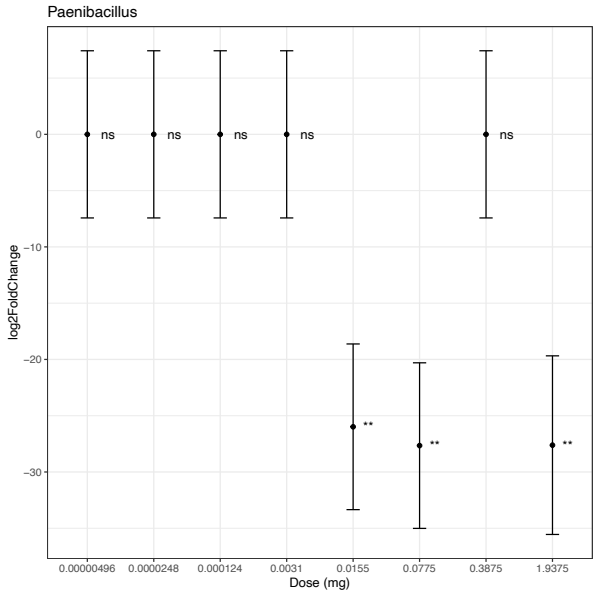

*Figure 22. Log fold changes (y-axis) in the different tested concentration of leachates (x-axis) compared to the non-exposed enrichments. "ns" represent the non-significant changes and "**" represents significant changes ($p<0.01$ respectively).*

3.4 The effects of ciprofloxacin on bacterial communities

3.4.1 Bacterial community growth

The growth curves of the ciprofloxacin exposure experiment were obtained from 88 wells (including controls and blanks) with four replicates per treatment. Although having a high degree of variation among the replicates, some trends could be identified, such as decrease in the OD measures under the highest antibiotic concentrations (figure 23).

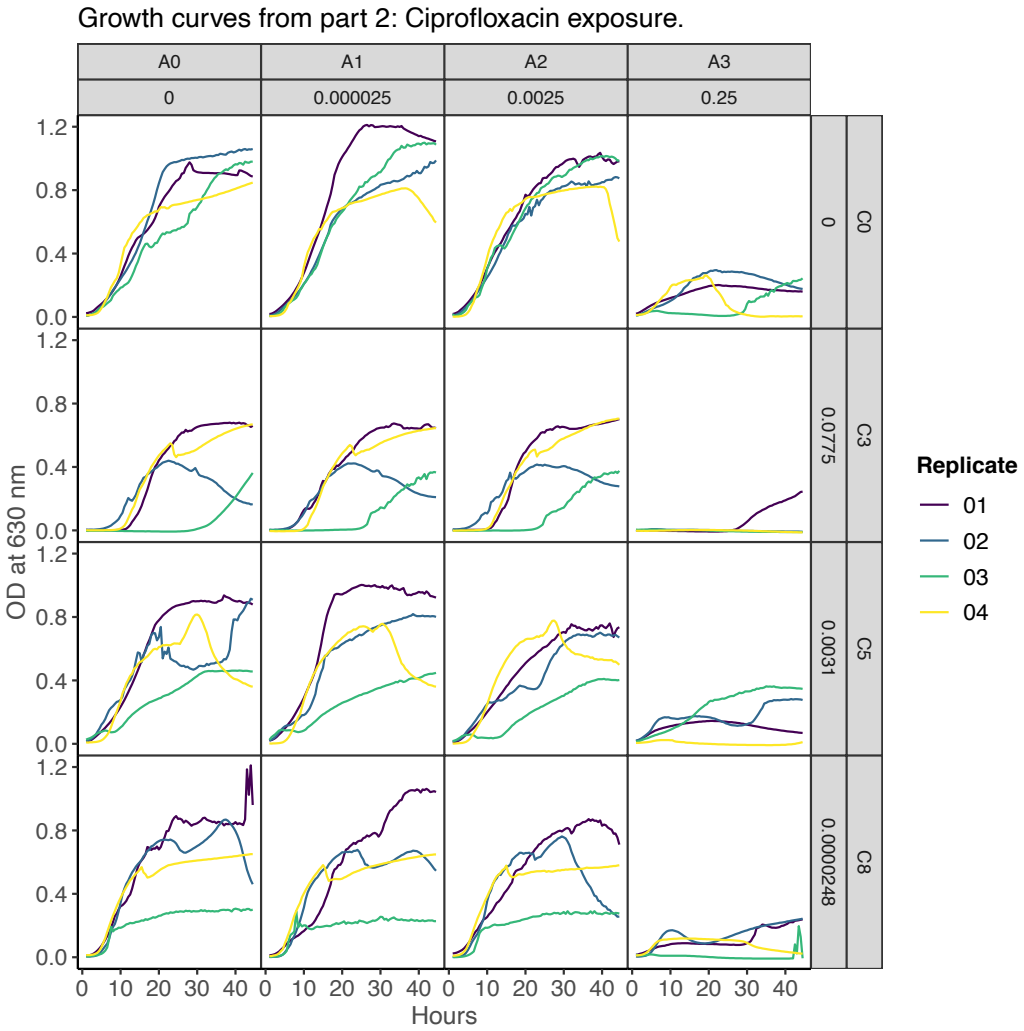

Figure 23. Growth curves obtained using optical density measurements at 630 nm from the ciprofloxacin experiment. Each box represents enrichments exposed to the specific leachate concentrations (mg/mL) indicated to the right, and ciprofloxacin concentrations (mg/mL) indicated above the boxes. The four different lines illustrate each of the four replicates per enrichment.

The yield (maximum optical density) ranged from 0 to 1.20, with a decline in yield in the highest concentration of antibiotics. By visual inspections of boxplots, unexposed bacterial enrichments yielded the highest maximum OD compared to preconditioned samples across

the antibiotic concentrations (figure 24). The linear maximum growth rates ranged from 0 to 0.19 OD/h, where the lowest growth rates were observed in the samples treated with the highest concentration of antibiotics. More intra-treatment variation was observed in the smoothed maximum growth rates, with the lowest at 0 and the highest rate at 0.43 OD/h. The lag phases were longest in the bacterial enrichments exposed to the highest leachate concentrations across antibiotics concentrations, however, for most of the samples, the lag phase increased with the leachate concentrations. The duration of the lag phases ranged from 0.35 hours up to 44.5 hours (the end of the incubations). The longest lag phases were observed in the samples treated with the highest concentration of antibiotics and leachates.

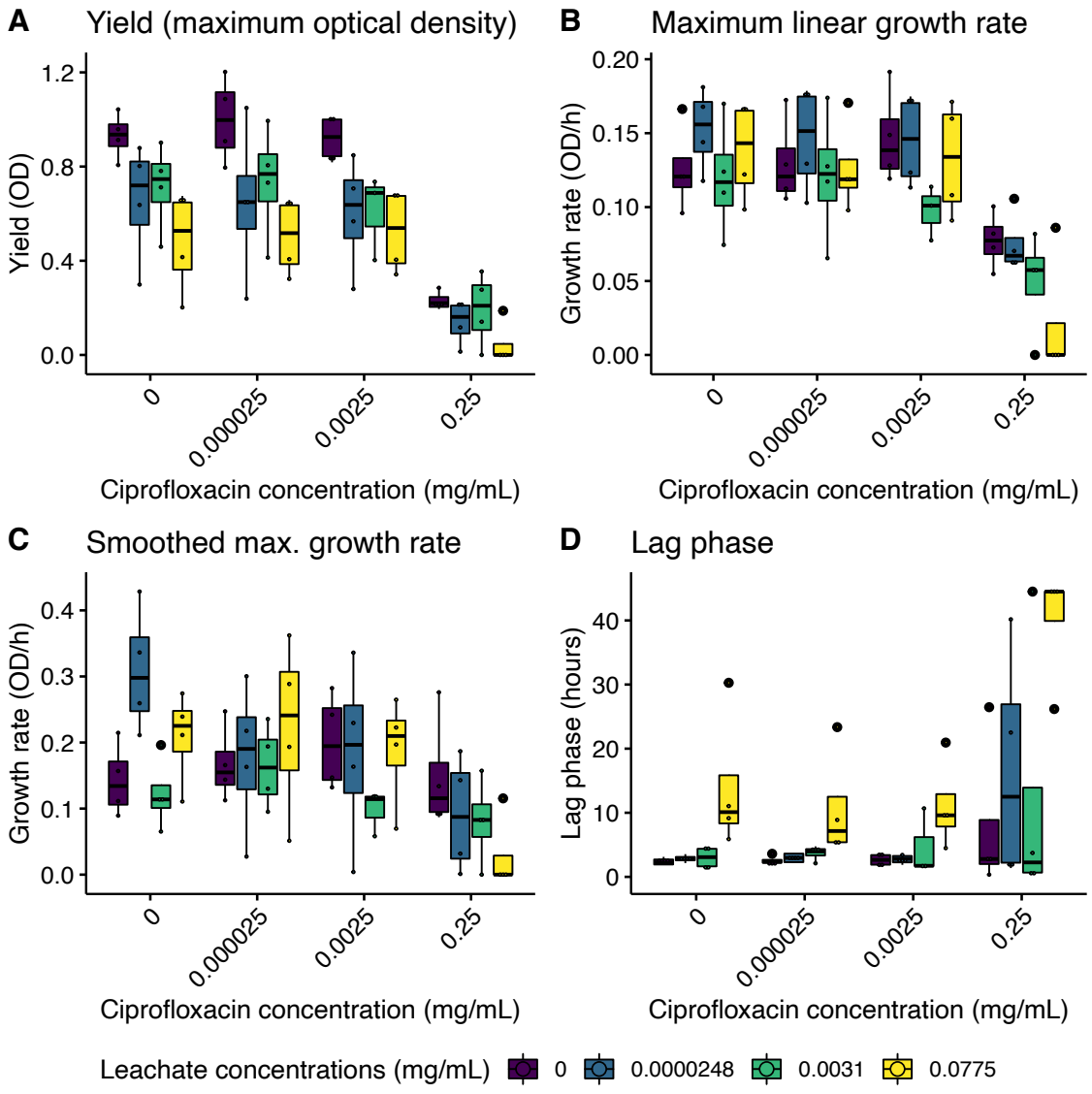

Figure 24. Boxplots of the observed growth curve parameters in response to ciprofloxacin exposure (mg/mL) on preconditioned enrichments. The dots represent each observation, with a total of four observations per treatment. The boxes represent the first and third quantiles, while the minimum and maximum values are represented by the whiskers. The thick, horizontal lines represent the median. The colours of the boxplots indicate the preconditioning concentration of leachates (mg/mL). Plot A illustrates the yield (maximum optical density) from each incubation. Plot B illustrates the maximum linear growth rates for each incubation. Plot C illustrates the maximum smoothed growth rates for each incubation, and plot D illustrates the lag phases from each incubation.

To predict effects of ciprofloxacin and leachates on yield (maximum OD) and maximum growth rates, linear models were fitted, while a generalised linear model with gamma distribution and identity link was fitted for lag phase. Leachate and ciprofloxacin concentrations explained a significant and substantial amount of the variation in the yield (R2 = 0.64, F(3, 59) = 34.57, p < 0.001, adj. R2 = 0.62). The intercept, indicating the response under no ciprofloxacin exposure and with no leachate exposure was at 0.77 OD (95% CI [0.70, 0.84], t(59) = 22.23, p < 0.001). Negative and significant effects of leachates and ciprofloxacin on maximum yield were identified (beta = -3.40, 95% CI [-5.16, -1.64], t(59) = -3.86, p < 0.001; Std. beta = -0.31, 95% CI [-0.47, -0.15]) and (beta = -2.31, 95% CI [-2.86, -1.76], t(59) = -8.46, p < 0.001; Std. beta = -0.73, 95% CI [-0.89, -0.58] respectively). A none significant, but positive interaction effect of ciprofloxacin and leachates was identified (beta = 6.29, 95% CI [-7.77, 20.35], t(59) = 0.90, p = 0.374; Std. beta = 0.07, 95% CI [-0.09, 0.23]). The yield had a lower starting point (representing no ciprofloxacin) in the incubations with the highest leachate concentration than incubations with lower leachate concentrations.

Leachate and ciprofloxacin concentrations explained a significant and substantial amount of the variation in the maximum growth rates (R2 = 0.55, F(3, 59) = 24.25, p < 0.001, adj. R2 = 0.53). Under no ciprofloxacin or leachate exposure, the intercept was at 0.13 OD/h (95% CI [0.12, 0.14], t(59) = 23.85, p < 0.001). Negative, but not significant effects were identified from changes in leachate concentrations (beta = -0.01, 95% CI [-0.30, 0.27], t(59) = -0.09, p = 0.931; Std. beta = -0.12, 95% CI [-0.29, 0.06]). Both ciprofloxacin and the interaction between ciprofloxacin and leachates had significant and negative effects (beta = -0.26, 95% CI [-0.35, -0.17], t(59) = -5.87, p < 0.001; Std. beta = -0.71, 95% CI [-0.89, -0.54]) and (beta = -2.39, 95% CI [-4.67, -0.12], t(59) = -2.11, p = 0.039; Std. beta = -0.19, 95% CI [-0.36, -9.33e-03]) respectively, meaning that increase in ciprofloxacin and leachates decreased maximum growth rates.

The model for smoothed maximum growth rates explained a significant and moderate amount of the variation in the maximum growth rates (R2 = 0.23, F(3, 59) = 5.87, p = 0.001, adj. R2 = 0.19). Under no ciprofloxacin or leachate exposure, the intercept was at 0.18 OD/h (95% CI [0.15, 0.21], t(59) = 11.59, p < 0.001). Significant and negative effects were identified from leachates (beta = -0.27, 95% CI [-0.52, -0.03], t(59) = -2.27, p = 0.027; Std. beta = -0.43, 95% CI [-0.66, -0.20]). Positive, but not significant effects of ciprofloxacin were identified (beta = 0.38, 95% CI [-0.40, 1.16], t(59) = 0.98, p = 0.330; Std. beta = 6.25e-03, 95% CI [-0.22, 0.23]). The interaction effect between ciprofloxacin and leachates was negative, but not significant (beta = -5.67, 95% CI [-11.87, 0.54], t(59) = -1.83, p = 0.073; Std. beta = -0.21, 95% CI [-0.44, 0.02]).

In the case of lag phases, leachate and ciprofloxacin concentrations could explain a substantial proportion of the observed variation with an intercept, representing unexposed incubations, at 2.81 hours (95% CI [2.17, 3.70], t(59) = 7.46, p < 0.001, Nagelkerke's R2 = 0.72). Leachates and ciprofloxacin induced positive and significant effects on lag phases (leachates: beta =

120.77, 95% CI [66.71, 213.98], t(59) = 3.50, p < 0.001; Std. beta = 6.07, 95% CI [3.26, 11.74] and ciprofloxacin: (beta = 36.99, 95% CI [20.23, 65.96], t(59) = 3.38, p < 0.001; Std. beta = 6.14, 95% CI [3.31, 11.88]). The interaction effect between leachates and ciprofloxacin was non-significant and positive (beta = 936.81, 95% CI [-175.67, 3514.47], t(59) = 1.18, p = 0.237; Std. beta = 3.44, 95% CI [-0.65, 12.91]). This is reflected in the regression lines with a higher starting point on the y-axis in samples treated with the highest concentrations of leachates. The regression lines have a trend to become steeper under high leachate concentrations in combination with increasing ciprofloxacin exposure, although the confidence interval is wide and covers the other leachate concentrations. See figure 25 for predictions of growth curve parameters.

Significant differences with large effects were identified between the antibiotic control and incubations without antibiotics in the following growth parameters: maximum yield (F(1, 6) = 357.88, p < 0.001; Eta2 = 0.98, 95% CI [0.94, 1.00]), smoothed maximum growth rate (F(1, 6) = 26.70, p = 0.002; Eta2 = 0.82, 95% CI [0.43, 1.00]), lag phase (F(1, 6) = 21126.35, p < 0.001; Eta2 = 1.00, 95% CI [1.00, 1.00]). Significant effects were identified in linear maximum growth rates (Kruskal-Wallis chi-squared = 6.0541, df = 1, p-value = 0.01387).

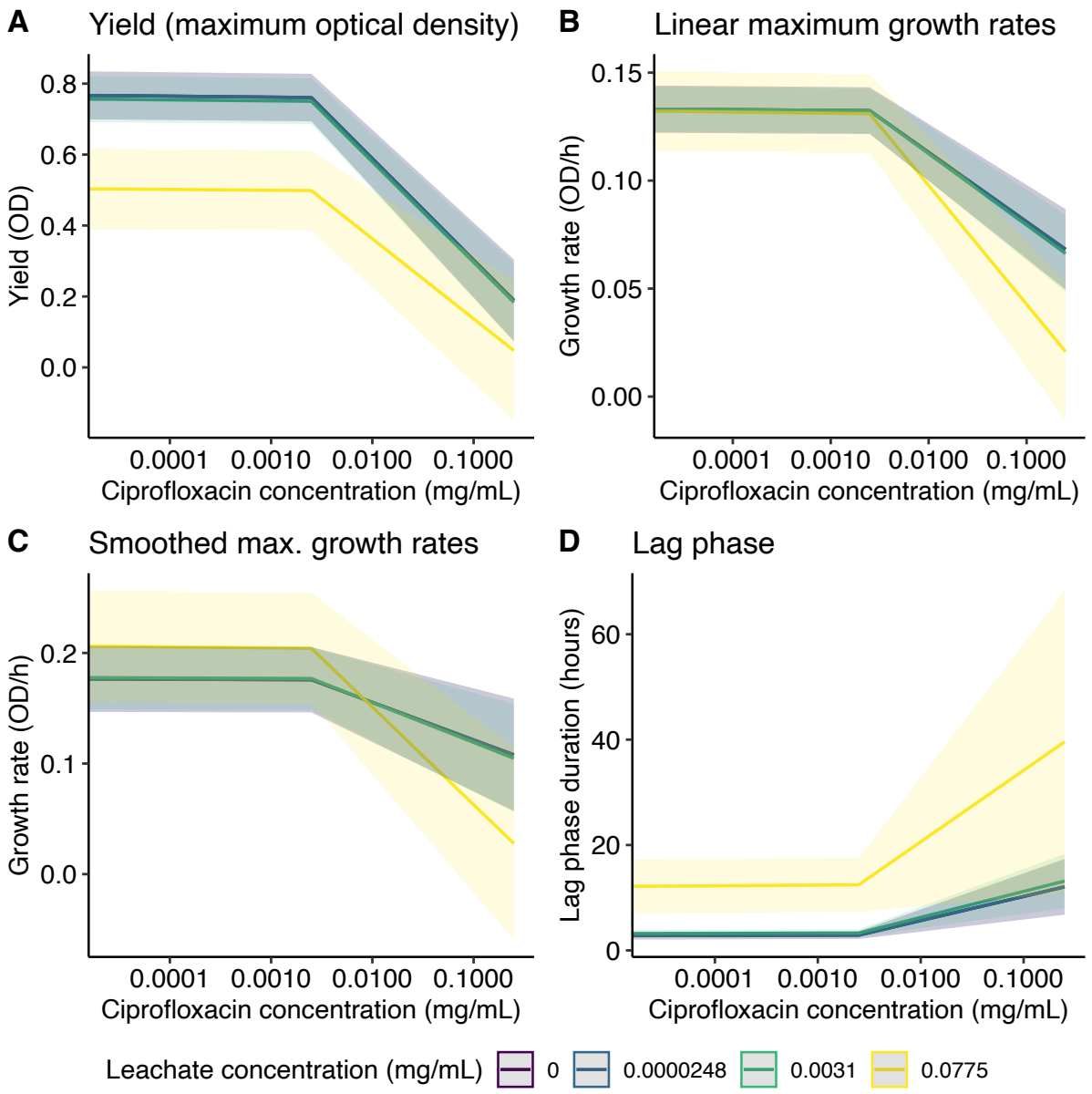

Figure 25. Predicted growth curve parameter- regressions in response to ciprofloxacin exposure (mg/mL) on preconditioned enrichments. Each regression line illustrates the different leachate concentrations the bacteria were preconditioned with (mg/mL), exposed to increasing ciprofloxacin concentrations. The transparent colour around the regression lines illustrates the corresponding confidence intervals. Plot A illustrates the yield (maximum optical density) from each incubation. Plot B illustrates the maximum linear growth rates for each incubation. Plot C illustrates the maximum smoothed growth rates for each incubation, and plot D illustrates the lag phases from each incubation. The predicted regression lines from the three lowest leachate concentrations are similar, being close to 0, resulting in overlapping regression lines and confidence intervals.

3.4.2 Bacterial community composition responses

The most abundant genera among all samples in the ciprofloxacin exposure experiment were *Bacillus*, *Psychrobacter*, *Paenibacillus*, *Paenisporosarcina* and *Burkholderia-Caballeronia-Paraburkholderia*. See figure 26 for overview of genera in all samples and proportion of reads in each sample.

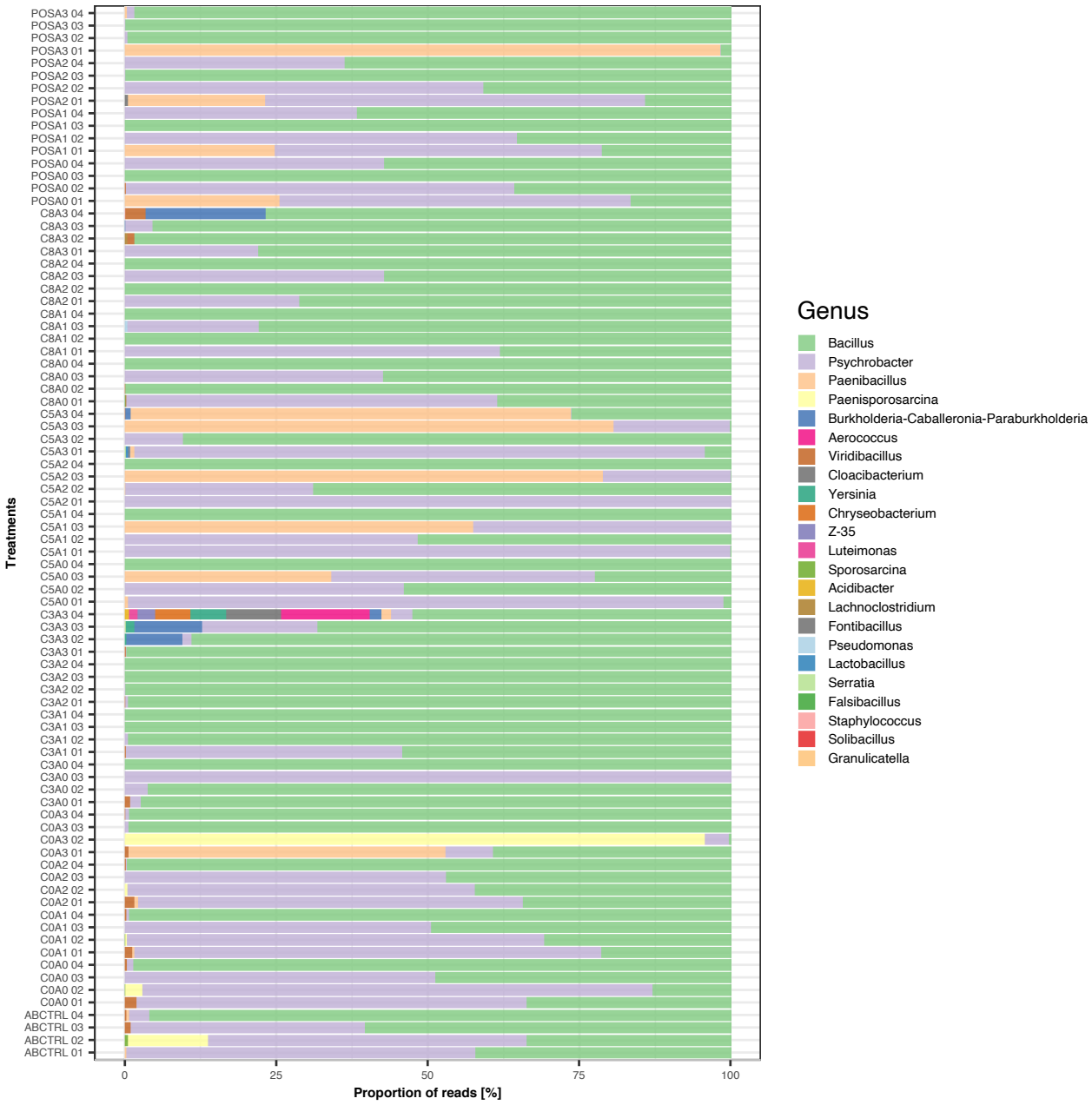

Figure 26. The proportion of reads of each bacterial genera in the different ciprofloxacin treatments. Each colour represents an individual genus. This plot was based on rarefied datasets. Samples named "C" represent the leachate preconditioning concentrations, while "A" represent the ciprofloxacin concentrations, where "A0" indicates no ciprofloxacin and "A3" indicates the highest ciprofloxacin concentration. Samples named "POS" represent the positive control from the leachate experiment. Samples named "ABCTRL" represent the enrichments exposed to the ciprofloxacin control. The number to the right (01-04) indicates the replicate.

In both alpha diversity indexes, no obvious trends relating to the different treatments were observable, other than the ACE richness appearing to be highest in unconditioned communities and at the highest ciprofloxacin concentrations (figure 27).

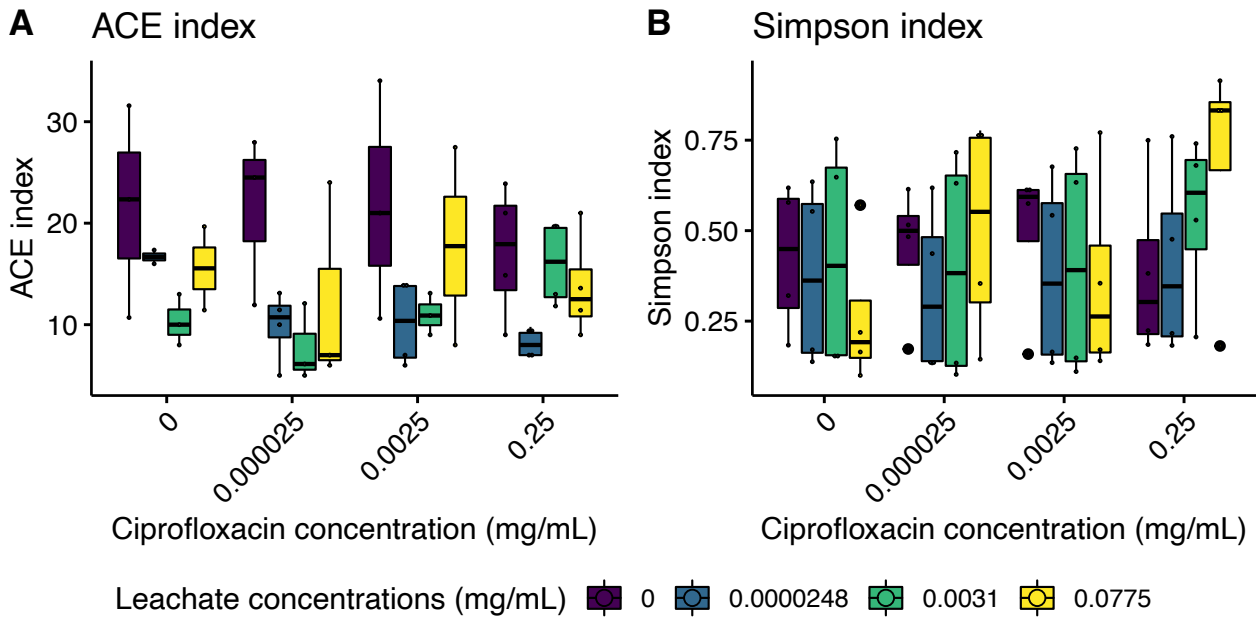

Figure 27. Boxplots of the observed alpha diversity metrics in response to ciprofloxacin exposure (mg/mL), resulting from the ciprofloxacin exposure on preconditioned enrichments. The dots represent each observation, with a total of four observations per treatment. The boxes represent the first and third quantiles, while the minimum and maximum values are represented by the whiskers. The thick, horizontal lines represent the median. The colours of the boxplots indicate the preconditioning concentration of leachates (mg/mL). Plot A illustrates the ACE index estimates, while plot B illustrates the estimated Simpson indexes.

The normality assumption was not met in the model for ACE richness, and although the homoscedasticity assumption was met some care must be taken when interpreting the predictions. The linear models to evaluate effects of the treatments of ACE richness and Simpson index were not significant (for ACE: R2 = 1.22e-03, F(3, 47) = 0.02, p = 0.996, adj. R2 = -0.06 and for Simpson index: R2 = 0.08, F(3, 60) = 1.69, p = 0.179, adj. R2 = 0.03). See figure 28 for predictions.

No effects of the positive control for antibiotics were observed in either of the alpha diversity parameters ACE index (F(1, 5) = 0.14, p = 0.720; Eta2 = 0.03, 95% CI [0.00, 1.00]) or Simpson index (F(1, 6) = 0.63, p = 0.457; Eta2 = 0.10, 95% CI [0.00, 1.00]) when testing against controls without any treatment.

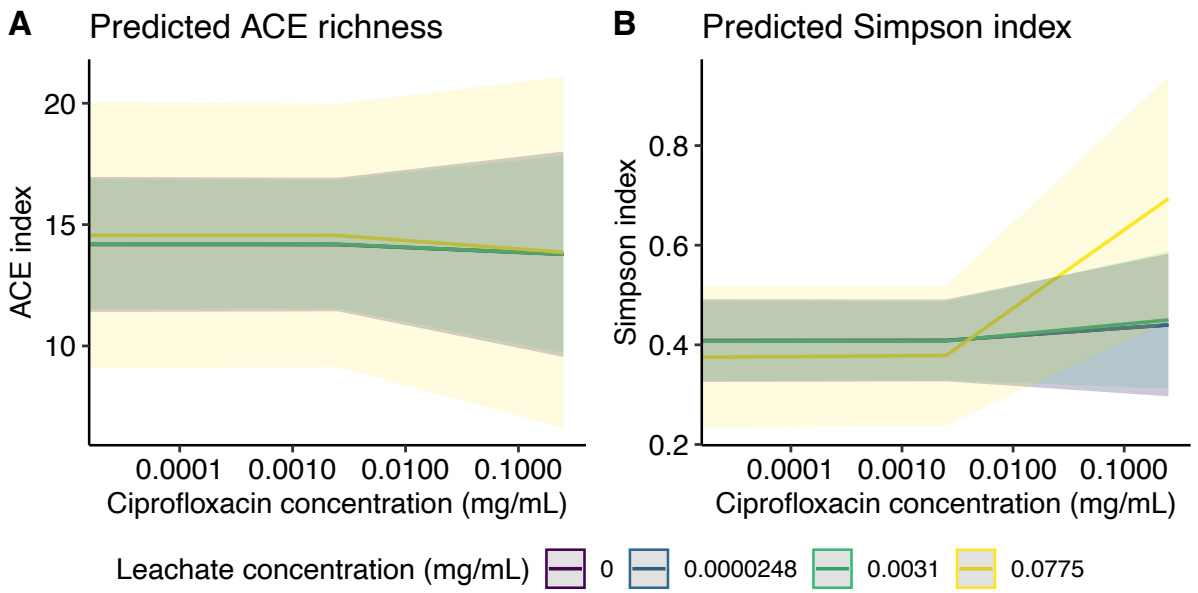

Figure 28. Predicted alpha diversity metric-regressions in response to ciprofloxacin exposure (mg/ml) on preconditioned enrichments obtained from linear modelling. Each regression line illustrates the different leachate concentrations the bacteria were preconditioned with, exposed to increasing ciprofloxacin concentrations. The transparent colour around the regression lines illustrates the corresponding confidence intervals. The colours of the regression lines indicate the preconditioning concentration of leachates (mg/mL). Plot A illustrates predicted ACE richness estimates. Plot B illustrates predicted Simpson index estimates. The predicted regression lines from the three lowest leachate concentrations are similar, being close to 0, resulting in overlapping regression lines and confidence intervals.

Beta diversity of the preconditioned bacteria exposed to ciprofloxacin was assessed for 64 samples. Visual assessment of NMDS-plot revealed potential effects of the preconditioning treatments on Bray-Curtis distances (figure 29). A clustering analysis based on Euclidian distances (figure 30) indicated some clustering based on leachate concentrations. This was confirmed by PERMANOVA, showing that the preconditioning with leachates produced significant differences (F(3) = 2.27, p-value = 0.003, R2 = 0.11, permutations = 999). No significant responses of either ciprofloxacin (F(3) = 0.63, p-value = 0.967, R2 = 0.03) nor the interaction effect of preconditioning and ciprofloxacin (F(9) = 0.35, p-value = 1, R2 = 0.05) were observed. Significant effects of leachates were found from a pairwise PERMANOVA, adjusted for multiple testing, between the following concentrations: C5 vs C8 (F(1) =3.54, p = 0.005, .adj = 0.04, R2 = 0.11), C5 vs C3 ((1) = 2.74, p = 0.036, .adj = 0.024, R2 = 0.08) and C8 vs C0 ((1) =3.10, p = 0.030, .adj = 0.04, R2 = 0.09). No significant differences resulting from leachates or ciprofloxacin exposure between the positive controls (3,5 DCP and ciprofloxacin) respectively were observed (leachates effect on 3,5 DCP: (F(1) = 0.81, p = 0.645, R2 = 0.075 and ciprofloxacin vs unexposed: (F(1) = 0.58, p= 0.981, R2 = 0.05).

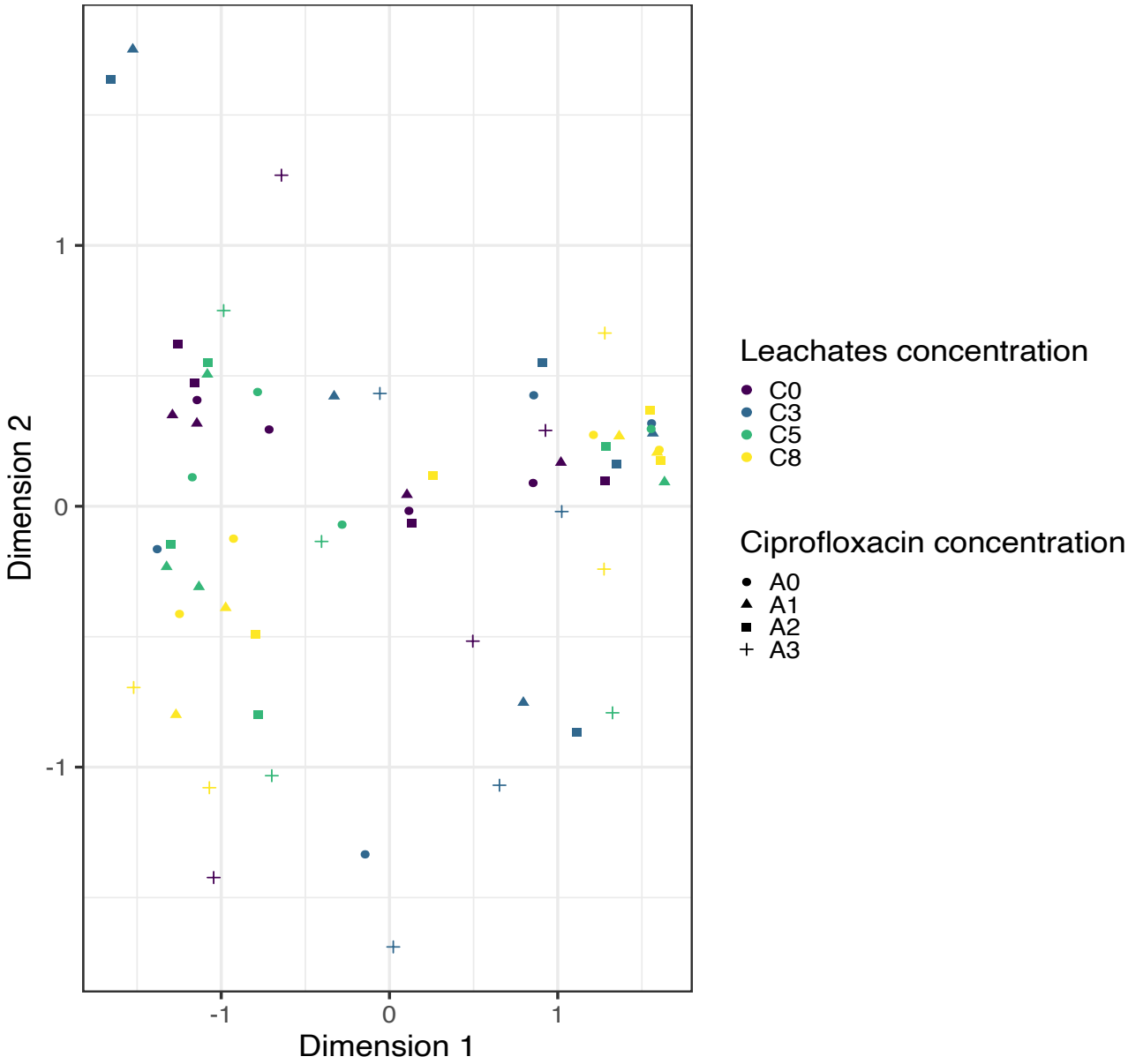

Figure 29. Beta-diversity in response to ciprofloxacin exposure on preconditioned enrichments visualised in a non-metric Multidimensional Scaling (NMDS) plot. Samples that cluster together indicate high similarity. The samples named "C.." indicate the concentration of leachates, with "C3" representing the highest leachate concentration and "C8" representing the lowest concentration of leachates, and "C0" indicating no leachate exposure. The "A" represents the ciprofloxacin concentrations, with "A0" representing no ciprofloxacin and "A3" representing the highest concentration of ciprofloxacin.

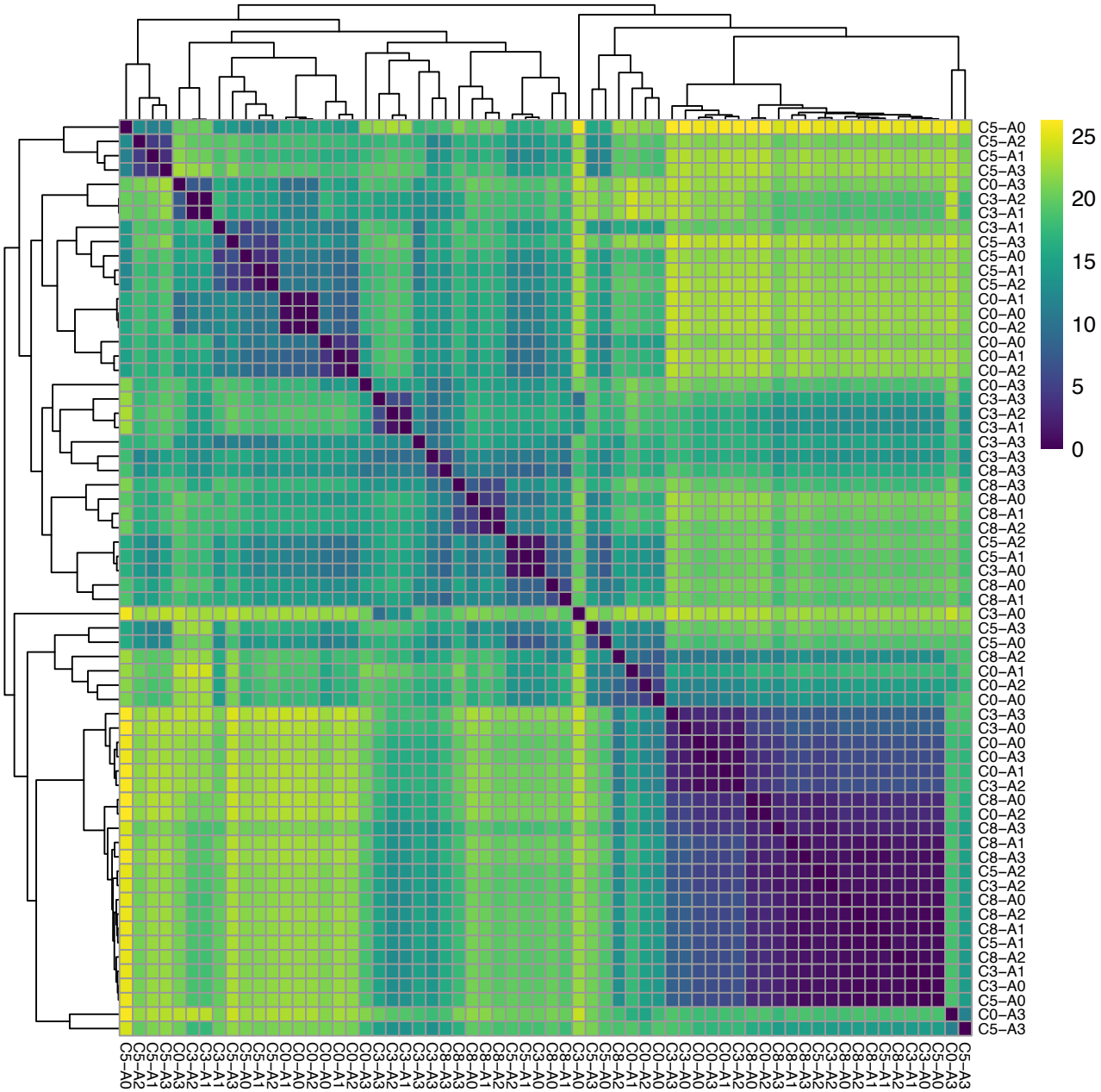

Figure 30. Beta diversity of the ciprofloxacin exposed enrichments visualised using Euclidean distances to visualise differences from treatments in the enrichments. The colour gradient illustrates the differences: squares coloured yellow represents the most distant samples, while the purple colour represents the most similar samples. The clustering of the samples is illustrated at the top and to the right. The samples named "C.." indicate the concentration of leachates, with "C3" representing the highest leachate concentration and "C8" representing the lowest concentration of leachates, and "C0" indicating no leachate exposure. The "A.." represent the ciprofloxacin concentrations, with "A0" representing no ciprofloxacin and "A3" representing the highest concentration of ciprofloxacin.

To identify changes in bacterial genera across the different treatments, a differential abundance analysis was performed using Deseq2. Comparisons were made between the different leachate concentrations and unconditioned bacterial communities within each dose of ciprofloxacin. See table 3 for overview of the comparisons. See figure 31 for an explanation for the plots.

Table 3. The comparisons between the leachate treatments ("C0"- "C8", see material and methods for concentrations) to assess significant log fold changes in ASVs in response to ciprofloxacin. The reference level is the "C0"-group of leachates (unconditioned enrichments) within each ciprofloxacin concentration ("A0"- "A3", see material and methods for concentrations).

A1.C3 vs A1.C0	A2.C3 vs A2.C0	A2.C3 vs A2.C0
A1.C5 vs A1.C0	A2.C5 vs A2.C0	A2.C5 vs A2.C0
A1.C8 vs A1.C0	A2.C8 vs A2.C0	A2.C8 vs A2.C0

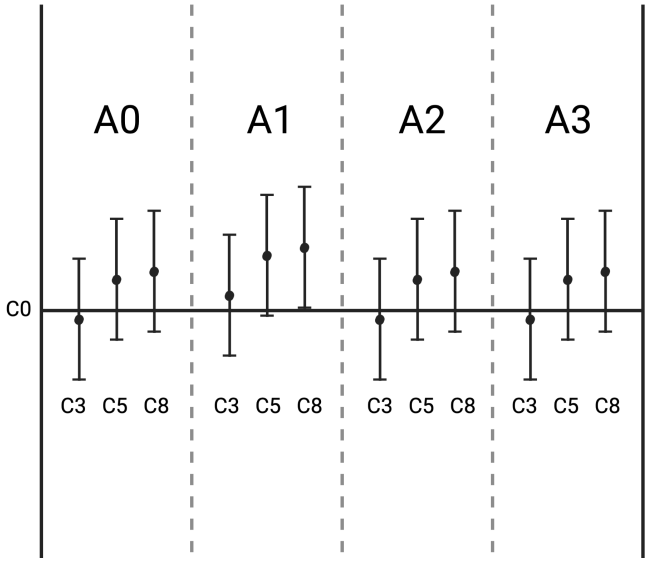

Figure 31. Illustration of the plots showing significant log fold changes (y-axis) in preconditioned ASVs (represented by "C3", "C5" and "C8") compared to unconditioned ASVs (represented by the horizontal line marked with "C0") within each concentration of ciprofloxacin (represented with "A0"- "A3") (x-axis).

In total 14 ASVs from three bacterial genera had significant log fold changes: *Paenibacillus*, *Psychrobacter* and *Bacillus*. Two *Paenibacillus* ASVs had positive log fold changes in leachate concentration "C5" under no ciprofloxacin exposure (see figure 32 for plot of one of the *Paenibacillus* ASVs). Three ASVs from *Psychrobacter* had significant negative log fold changes under ciprofloxacin exposure: "A2C3" (two) and "A1C8" (one). One *Psychrobacter* ASV had significant positive log fold change under ciprofloxacin: "A3C5" (see figure 33 for example of *Psychrobacter* plot). ASVs from *Bacillus* had both positive and negative significant log fold changes (figures 34 and 35), although none of these changes were under ciprofloxacin exposure. The negative log fold changes were found in "A0C5" (six ASVs) and "A0C3" (one),

and the positive log fold change was found in “A0C5”. See the appendix for all plots of genera with significant log fold changes.

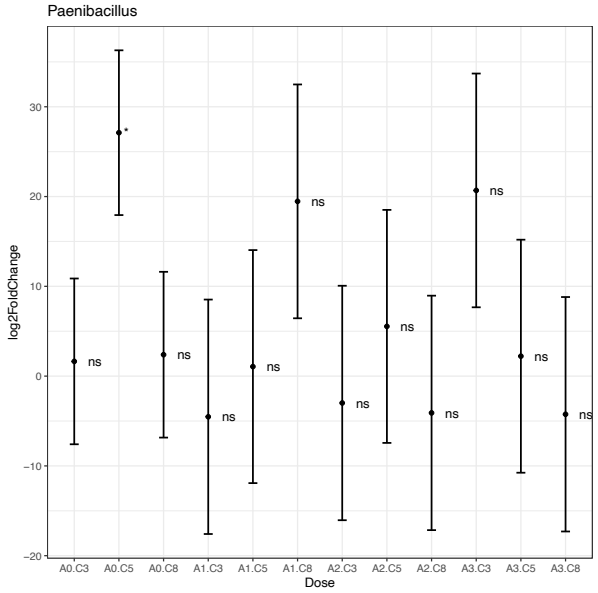

Figure 32. Log fold changes in ASVs (y-axis) in preconditioned enrichments compared to the unconditioned enrichments within each ciprofloxacin concentration (x-axis). “ns” represent the non-significant changes and “” represents significant changes (p<0.05).*

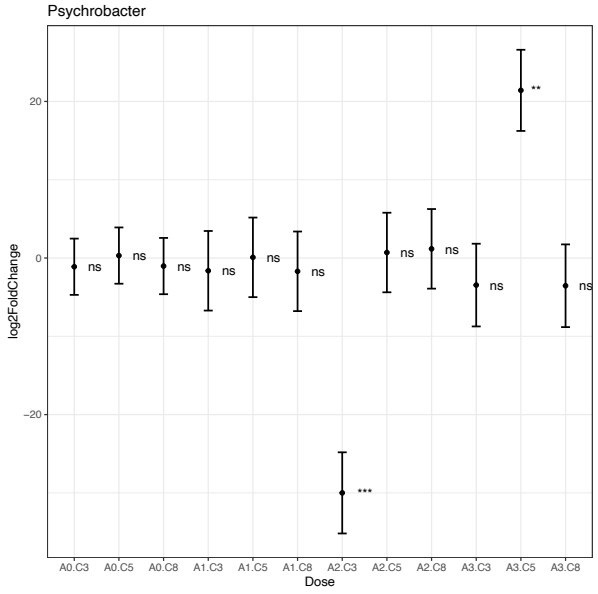

*Figure 33. Log fold changes in ASVs (y-axis) in preconditioned enrichments compared to the unconditioned enrichments within each ciprofloxacin concentration (x-axis). “ns” represent the non-significant changes, “**” and “***” represents significant changes (p<0.01 and p<0.001 respectively).*

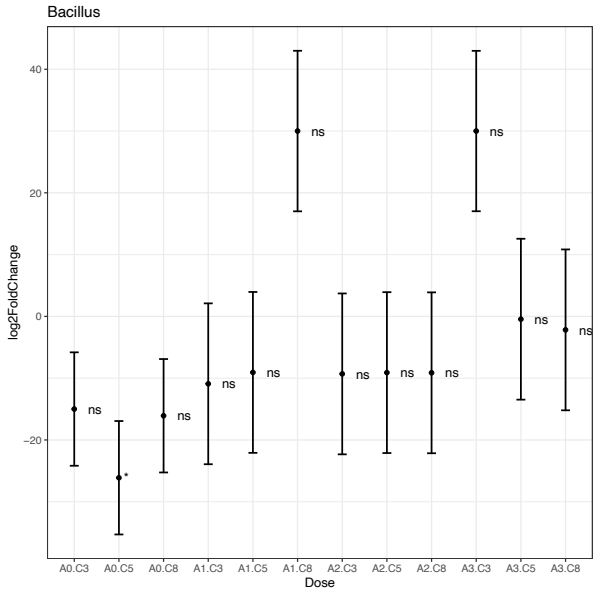

Figure 34. Log fold changes in ASVs (y-axis) in preconditioned enrichments compared to the unconditioned enrichments within each ciprofloxacin concentration (x-axis). "ns" represent the non-significant changes and "" represents significant changes (p<0.05).*

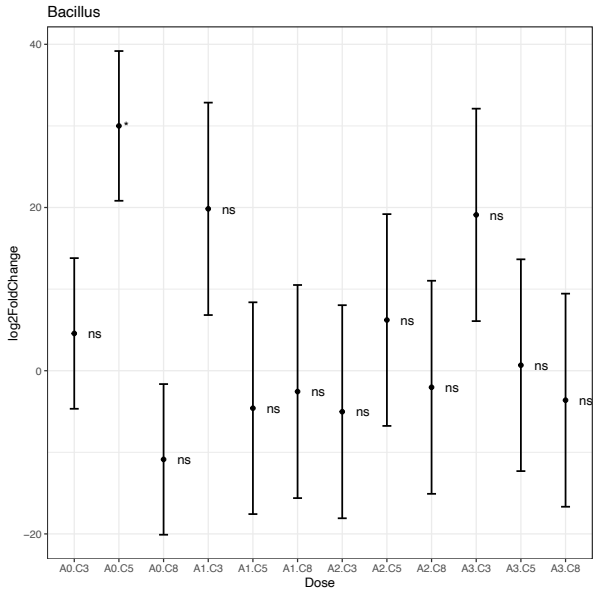

Figure 35. Log fold changes in ASVs (y-axis) in preconditioned enrichments compared to the unconditioned enrichments within each ciprofloxacin concentration (x-axis). "ns" represent the non-significant changes and "" represents significant changes (p<0.05).*

4 Discussion

4.1 Effects of leachates on bacterial communities

4.1.1 Bacterial community growth responses

All growth curve parameters were significantly and negatively affected by leachate exposure at concentrations between 0.0155mg/mL and 0.0775 mg/mL. At concentrations above 0.0775 mg/mL a total absence of growth was observed, indicating that the leachates from washing gloves have the potential to inhibit the growth of complex microbial communities. Similarly, impaired microbial growth resulting from different types of plastic material leachate exposure has been identified in various microorganisms under different test conditions (Capolupo et al., 2020; Hjelset, 2021; Klein et al., 2021; Qiu et al., 2022; Sarker et al., 2020; Schiavo et al., 2021; Tetu et al., 2019). Other adverse effects of leachates on microorganisms have been identified, such as mutagenicity and genotoxicity, and inhibition of bioluminescence in a Microtox assay (Alabi et al., 2019; Zimmermann et al., 2019), while others find no adverse effects; rather stimulated growth has been observed in microalgae (Chae et al., 2020; Piccardo et al., 2020).

The effects of the leachates on bacterial growth may arise from some of the substances identified in the leachates. A need for more focus on leachate composition for better interpretation of the toxicological results have been raised (Delaeter et al., 2022). As such, a brief assessment of the chemicals present in our study, resulting from primary plastic leaching, has been conducted. Although identifying many chemicals, studies have shown that a large amount of chemicals present in plastic leachates are unknown, and unidentifiable when using the NIST database as reference library (Zimmermann et al., 2019), as was done for the leachates in this project. In the washing gloves leachates, substances, which are known to induce adverse effects on bacteria, were identified and include benzenamine (National Center for Biotechnology Information, 2022), 2-mercaptobenzothiazole (ECHA, 2022b) and zinc (ECHA, 2022f). Some of the substances were found to not induce adverse effects in microorganisms except at environmentally unrealistic high concentrations (> 100 mg/L), such as 2,4,7,9-tetramethyl-5-decyn-4,7-diol (ECHA, 2022a), heptadecane (ECHA, 2022e) and 1-hexadecanol (ECHA, 2022c). Concentrations of substances associated with micro-and nano plastics range in concentrations from a few pg/L to many µg/L in marine waters worldwide (Gunaalan et al., 2020; Hermabessiere et al., 2017).

Our results indicate that the bacterial community growth was adversely affected by high concentrations of leachates only. It was previously shown that concentrations of substances associated with micro-and nano plastics ranged from a few pg/L to many µg/L in marine waters worldwide (Gunaalan et al., 2020; Hermabessiere et al., 2017). However, plastic debris function as new ecological niches on which microbial communities attach themselves and

reside (Zettler et al., 2013). Such communities, referred to as the “plastisphere”, are distinct from surrounding seawater (Zettler et al., 2013). On a microscale, microorganisms in the plastisphere may be exposed to high concentrations of plastic leachates in the immediate vicinity of plastics due to diffusion gradients (Rochman et al., 2014). In addition, leachates are complex mixtures, and these mixtures of substances can have additive effects (Roose-Amsaleg et al., 2021; Tsiridis et al., 2006). There is clearly a need for more research on mixture toxicity, as compared to single substance toxicity (Groh et al., 2019; Gunaalan et al., 2020).

A further point needed to be addressed, although it was beyond the scope of this project, are effects of substances in the environment that may adsorb onto plastics (Brennecke et al., 2016; Hirai et al., 2011; Li et al., 2018; Teuten et al., 2009). The gloves used in this study have only been subject to laboratory testing, so the consequences of plastic weathering in the environment are not represented. Such substances that adsorb to the plastics may be of environmental concern (Koelmans et al., 2016; Larue et al., 2021; Velzeboer et al., 2014; Vila-Costa et al., 2020; Yu et al., 2019), and bacteria may be exposed to high concentrations in the plastisphere. This illustrates the complexity of environmental concerns in relation to plastic. A further challenge is the knowledge gap on possibly adverse effects of many of the chemicals identified in the leachates used in this study; little to no ecotoxicological assessment regarding microorganisms has been conducted to the best of our knowledge on these substances (searches were done in the databases of PubChem and ECHA on April 29th, 2022). Such a lack of ecotoxicological research has been identified by others as well: (Capolupo et al., 2020; Groh et al., 2019; Gunaalan et al., 2020; Romera-Castillo et al., 2018; Schiavo et al., 2020; Tetu et al., 2019).

While studies have shown that sea urchins and their development may be adversely affected by plastic leachates (Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Rendell-Bhatti et al., 2021), no research has to our knowledge been conducted on the effects of leachates on their microbiome. Being in the gut of sea urchins, the microbiome can be expected to be exposed to plastic associated chemicals, resulting from increased release of leachates when plastics are exposed to digestive fluids. Gunaalan et al. (2020) proposed increased focus on this possibility of leaching mechanism from plastics. Thus, adverse effects on the sea urchin microbiome, such as the inhibition of growth observed in this project, could possibly result in effects on the urchin health and possibly affect ecologically important ecosystem services from urchins such as bioturbation in sediments. As such, plastic leachates may have severe consequences on the ecosystems, although assessing effects on sea urchin health is beyond the scope of this project.

4.1.2 Bacterial community composition responses to leachates

The most abundant species in the leachate experiment were *Bacillus*, *Psychrobacter*, *Paenibacillus*, *Lysinibacillus*, *Sporosarcina* and *Bacteroides*. All these bacterial genera have been observed either in marine environments or in associations with marine invertebrates

(Ivanova et al., 1999; Menezes et al., 2010; Romanenko et al., 2002; Sfanos et al., 2005; Wang et al., 2021). Increase in the alpha diversity measures ACE richness (increase in total number of species) and Simpson index were observed at high leachate concentrations. Seemingly contradictory, the Simpson index (not to be mistaken for the Simpson diversity index) puts most weight to the most abundant species in the samples. Thus, a decrease in the most abundant samples, being replaced by a higher number of less abundant species, could result in an increase in Simpson index, as observed in this experiment. This indicates that the leachates may have inhibited the growth of some dominant species, allowing opportunistic, but low abundant species to be observed. Such a change in the community assembly has been observed in an exposure study of environmentally significant concentrations of anthropogenic dissolved carbon (ADOC) (including chemicals from plastic leachates) on bacterial communities (Cerro-Gálvez et al., 2019). In this study, ADOC exposure led to an increase of rare marine bacteria (Cerro-Gálvez et al., 2019). Other studies have also identified differences in sensitivity of bacterial taxa to the chemicals in plastic leachates, possibly promoting such community assembly changes. However, no effects in beta-diversity were observed in response to leachate exposure, indicating that the different bacterial communities share many bacterial genera even after different leachate exposure.

Of the bacterial genera observed, only ASVs from two genera showed significant increased log fold changes in response to leachate exposure, *Psychrobacter* and *Bacillus.* Still not all ASVs belonging to these genera exhibited equal responses. This is similar to the results by Tetu et al. (2020) who investigated effects of low concentration leachates on different strains of the cyanobacteria *Prochlorococcus* and identified that sensitivity to chemicals differed among the strains. Thus, they proposed that some strains may "win" over other strains in areas with high accumulations of plastic litter (Tetu et al., 2020), possibly causing community assembly changes. Other possible explanations for alterations in community assembly have been proposed by Tetu et al. (2020); if plastic leachate-metabolising bacteria are present, they might reduce the potential adverse effects on other more sensitive microorganisms present in the community, or the cell lysis of sensitive bacteria following plastic leachate exposure may result in increased nutrition (in the form of organic matter) for the resistant bacteria. In accordance with this, increased bacterial activity has also been observed from plastic leachate exposure (Birnstiel et al., 2022; Romera-Castillo et al., 2018). The diverse responses to leachates of *Bacillus* ASVs could be due to difference in metal resistance, as observed previously for some *Bacillus* strains being resistant to zinc at toxic concentrations (Podlesek et al., 1993), one of the metals identified in the leachates in this experiment. Other studies have also shown that certain strains of *Bacillus* species and their spores may be resistant to different chemicals (Vilas-Bôas et al., 2007), illustrating their possible resilience towards leachates.

In most other cases when bacterial ASVs showed significant log fold changes these were negative log fold changes. These included ASVs from the genera *Sphingomonas*, *Lactococcus*,

Alkalibacterium, *Paenibacillus* and *Sporosarcina* and even *Bacillus*. The most sensitive ASVs were found to belong to the genus *Bacillus*, being negatively affected already at the lowest concentration of leachates, while the ASVs from other genera were affected only at high concentrations (from 0.0155 mg/mL and above). This could indicate that some *Bacilli* ASVs are more sensitive to the leachates than the other bacterial ASVs.

This experiment has shown that the impacts of plastic leachates on marine bacteria are complex and may promote bacterial growth and activity in some instances, while inhibiting growth and activity in other circumstances. Such diverse effects may be ascribed to differences in bacterial adaptations, such as the ability to efflux toxic compounds out of the cell, or complex bacterial community interactions, where the survival of some bacterial strains may affect the survival of other bacteria. Other explanations for the diverse responses are the chemical composition and concentrations of plastic leachates, which may include toxic substances, complex chemical mixtures, or substances metabolizable by bacteria, inducing diverse bacterial community responses at different leachate concentrations.

4.2 Effects of ciprofloxacin on bacterial communities preconditioned with leachates

4.2.1 Bacterial community growth responses

Significant decrease in yield and prolongation of lag phases were observed in highly preconditioned enrichments under no ciprofloxacin exposure, corroborating the findings of the preconditioning experiment, that leachates alone can decrease the growth of the enriched gut microbiome community. The prolonged lag phase possibly indicates that more energy may be spent on excreting the leachates from the bacterial cytosol, before exponential growth could be initiated, thus resulting in less energy being spent on growth. Such energy allocation has been identified in bacteria using efflux pumps to remove xenobiotics from their cytosol, where high concentrations of such substances may induce increased expression of efflux pumps, at the expense of other metabolic functions (Martinez et al., 2009), or resulting in efflux of vital nutrition (Webber & Piddock, 2003).

Furthermore, significant negative effects of ciprofloxacin were observed in yield and maximum growth rates as approximated by linear growth models, and lag phase was significantly prolonged by ciprofloxacin, indicating that the bacterial communities were affected by ciprofloxacin at concentrations above 1 mg/L. This concentration was, however, much higher than the concentrations of ciprofloxacin identified in aquatic environments, ranging up to 400 ng/L (Grenni et al., 2018; Miao et al., 2004), indicating that environmentally significant concentrations of ciprofloxacin may not inhibit the growth of the bacteria present in this experiment.

4.2.2 Interaction effects between leachates and ciprofloxacin

A significant interaction effect between leachates and ciprofloxacin was observed for maximum growth rates as approximated by linear growth models only, showing an enhanced decrease in growth when exposed to both, indicating possible synergistic effects between leachates and ciprofloxacin. As we did not observe any significant degressive interaction effects between leachates and ciprofloxacin on any of the growth parameters, it can be assumed that preconditioning with leachates prior to ciprofloxacin exposure did not lead to increased community adaptation to antibiotics. If efflux pumps were induced by leachates, possible explanations for the lack of increased ciprofloxacin adaptations includes that ciprofloxacin may not have been transported by the same pumps, or that the physiological cost of overexpression of the pumps was superior to the benefits of removing the antibiotics from the cells. Overexpression of efflux pumps transporting a variety of substances in bacteria have been shown to negatively affect their physiology (Alonso et al., 2004). As such, rather than decreased susceptibility to antibiotics, the interaction between leachates and ciprofloxacin seemed to inhibit bacterial growth in this experiment.

4.2.3 Bacterial community composition responses

The number of bacterial genera decreased from 63 after leachate exposure to 23 after subsequent ciprofloxacin exposure, with the most abundant bacteria genera being *Bacillus, Psychrobacter*, *Paenibacillus*, *Paenisporosarcina* and *Burkholderia-Caballeronia-Paraburkholderia* after leachate exposure. However, although present in the leachates experiment, *Paenisporosarcina* and *Burkholderia-Caballeronia-Paraburkholderia* became more abundant after ciprofloxacin exposure relative to the genera present. This change could indicate that these genera are more tolerant to ciprofloxacin than other genera. *Paenisporosarcina* species are known to produce endospores (Reddy et al., 2013), which could be initiated in the presence of ciprofloxacin, making it possible to identify the species through metabarcoding. The reduction in number of species could indicate susceptibility to ciprofloxacin among many genera, thus corroborating the bacterial growth parameter findings. *Bacillus* species were more abundant in the ciprofloxacin experiment than in the leachates experiment, while the opposite is true for *Psychrobacter* species, possibly indicating differences in susceptibility towards leachates and ciprofloxacin among them.

To further understand the community structure composition, the alpha diversity metrics can provide information regarding potential shifts in species richness or evenness in response to leachates and ciprofloxacin. However, in our study, there was an absence of significant alpha diversity changes in response to ciprofloxacin after leachate preconditioning. Furthermore, the complexity of community assembly, as described in the discussion of leachate exposure, could possibly explain why no such significant differences were observed. A somewhat higher richness was observed in the unconditioned bacteria in all ciprofloxacin concentrations, and although not being significant, it could possibly indicate that these bacteria were optimised

for the growth conditions in our experiment, and that fewer species survived combined leachate and ciprofloxacin exposure. The beta diversity was only significantly affected by leachate concentrations, indicating that preconditioning with leachates led to differences in community assembly. However, no effects of ciprofloxacin were observed on beta diversity, possibly indicating that leachate exposure does not select for bacterial communities with decreased susceptibility to antibiotics.

As in the case of significant log fold changes in preconditioned bacteria under ciprofloxacin exposure, the ASVs from the *Psychrobacter* genus with negative changes under ciprofloxacin could indicate that *Psychrobacter* may be sensitive to antibiotics. Interestingly, one ASV from this genus experienced increased log fold changes under the highest ciprofloxacin concentration under moderate ("C5") leachate exposure, possibly indicating increased resistance towards antibiotics. Studies have identified genes that regulate resistance towards toxic compounds and antibiotics in *Psychrobacter*, such as multidrug resistant efflux pumps and fluoroquinolone resistant genes (Raghupathi et al., 2016). However, possible responses to ciprofloxacin were only observed in one *Psychrobacter* ASV, which may indicate that intermediate concentration of leachate may be the optimum preconditioning dose for developing resistance against antibiotics. However there are multiple other explanations than cross-resistance that could explain these results. In fact, even though resistance towards ciprofloxacin mediated through efflux pumps has been observed in *Bacillus* (Price et al., 2003), *Pseudomonas* (Pagedar et al., 2011; Rehman et al., 2019) and *Staphylococcus* species (Hassanzadeh et al., 2017; Webber & Piddock, 2003), the only positive log fold changes in *Bacillus* ASVs in this study were observed in enrichments without ciprofloxacin exposure, and no significant log fold changes were observed in *Pseudomonas* or *Staphylococcus* ASVs. Although *Paenibacillus* species have been observed to tolerate high concentrations of trace metals, such as zinc (Orji et al., 2021) which was identified in the leachates of the current project, the significant positive log fold changes in response to intermediate concentrations of leachates in two *Paenibacillus* ASVs could indicate a tolerance towards leachates but not ciprofloxacin, indicating no common resistance mechanism towards the two substances. As such, no indications for increased resistance against ciprofloxacin following preconditioning with leachates was evident in our experiment.

Another possible explanation for the lack of increased resistance, could be that the intervals between the concentrations of leachates were too coarse. A threshold effect was observed in the leachate exposure experiment, so by focusing on concentrations in the range between 0.0031-0.3875 mg/mL, more sensitive responses may have been observed, thus possibly inducing increased adaptations to ciprofloxacin. In addition to this hypothetical explanation, the lack of increased resistance could be because of bacteria in the enrichments using different efflux pumps for the leachates, than the efflux pumps involved in ciprofloxacin removal, or completely other mechanisms to cope with leachate exposures. The identification of resistance mechanisms to both leachates and ciprofloxacin were beyond the scope of this project but assessing metagenomics and proteomics in a similar experiment would allow for

identification of present genes (with a possible focus on genes encoding for efflux pumps taking a wide range of substrates, including xenobiotics), and to identify potential differences in expression of those genes (W. Liu et al., 2020; Ma et al., 2021). Such an approach would allow for deeper insight into the mechanisms behind possible adaptations toward antibiotic resistance.

4.3 Future challenges and prospects

A limitation of this study was the use of optical density, being a measure of turbidity, for estimating the various community growth estimates. The optical density is often interpreted to be correlated with either bacterial numbers or concentrations, although differences in bacterial cell size and differences in light scattering arising from cell size and shape (as a result of community change) or differences in growth media may affect the readings (Stevenson et al., 2016). Cell counting could calibrate for such challenges, this would also allow counting only cells containing DNA and/or RNA (Stevenson et al., 2016). However, this would include extracting several samples of the incubations for viable cell counting, leaving nothing left for DNA analysis. On the other hand, as antibiotics may induce filamentation, the relationship between OD and bacterial concentrations becomes less evident, and may no longer correlate without calibration (Stevenson et al., 2016). Differences in bacterial morphology, expected to occur in a bacterial community consisting of many different bacterial genera, may affect the OD measurements, resulting in unreliable growth curve measurements. The same may be true in the presence of ciprofloxacin, an antibiotic which is known to induce filamentation in bacteria (Stevenson et al., 2016). However, this challenge was addressed by analysing the community assembly through DNA metabarcoding, in which identification of bacterial genera in the different samples provide insight into possible community assembly changes.

Another challenge relating to the differences in cell size is that if bacteria with large cell size "win" over bacteria with smaller cell size, the resulting OD measurements may indicate higher cell doublings (maximum growth rates) than actually being the case. A possible solution to this challenge could be to assess one bacterial strain expected not to have a large increase or reduction in cell size during growth, or use viable count, to assess whether preconditioning with leachates may induce adaptations to ciprofloxacin. However, this would remove aspects of community interactions that we wanted to account for in this experiment.

The decrease in growth/prolonging of lag phase or change in diversity in this experiment may also result from competition and other ecological functions, and it is therefore not possible to determine if preconditioning with leachates alone is the only driver behind increased or decreased growth / diversity. Selection, diversification, and drift are processes that in addition to or instead of leachates could explain patterns in community assembly. Selection on bacteria with beneficial traits may lead to the observed community assembly (Nemergut et al., 2013). For instance, bacteria that metabolises substances present in the enrichments have an

advantage over bacteria unable to do the same, leading to potential increased selection on the former. Selection on specific traits possibly acquired through horizontal gene transfer may also form the community (Burke et al., 2011). Bacteria in the vicinity of others with beneficial genes would have an advantage over bacteria further away, resulting in increased growth of bacteria in that specific location. Another way the community assembly may change is through diversification (Nemergut et al., 2013). Diversification can arise from the presence of dormant bacteria, in which species respond to the stress by inducing spore-production, awaiting activity until restoration of more favourable conditions (Jones & Lennon, 2010; Nemergut et al., 2013). An example of stress may be exposure to leachates and/or ciprofloxacin. In such a situation, it would be possible to observe increased species richness through metabarcoding analysis without observing increased growth, as was observed in the leachate experiment in this study. Finally, drift, the process in which random changes in the community abundance occur (Nemergut et al., 2013), could result in the loss or gain of important species, either inducing or reducing growth in the microbial community. In addition to these three main processes, competition, for instance through the creation of antimicrobial agents, and predation among bacterial species may explain why some bacteria become more abundant than others (Hibbing et al., 2010), and why some enrichments have higher growth rates and yields than others. All these processes illustrate the complexity of bacterial communities, and the challenges facing us when trying to disentangle ecological consequences of plastic pollution.

Other limitations in the present study include the bacteria in these experiments being limited to those growing on the chosen medium, which may not sufficiently replicate the natural nutrition in the sea urchin gut. Freezing and thawing of bacterial enrichments could also favour the resilient bacteria (Morley et al., 1983), making us miss the effects on sensitive bacterial genera. The experimental design allowed for relatively simple statistics, making interpretation of results straight forward. However, an increase in replicates would enhance the power of the statistics, which was evidently needed for alpha diversity metrics in the ciprofloxacin experiment, where the confidence intervals were broad, possibly resulting from larger intravariation than intervariation among samples. In addition, the PCR-amplification method used in this study allows only for semi-quantitative analysis of community assembly (Angly et al., 2014; Gardham et al., 2014), thus some care must be taken when assessing the alpha and beta-diversity metrics. However, metabarcoding allows for detecting more sensitive end-points (Yang et al., 2018; Zhang et al., 2018), making it possible to observe bacterial community composition changes impossible to assess with more traditional methods, such as metabolic assays or microscopy (Martin et al., 2020).

The results of the present study cannot be generalised to all sea urchins, as bacteria from only one urchin was used. Furthermore, no health effect end-points for sea urchins were assessed. However, an advantage of using enrichments as model systems to study toxic effects includes the possibility to limit the number of parameters that may affect the bacterial responses *in vivo* (Blasche et al., 2017). Such an approach facilitates studies exploring the effects of

leachates and ciprofloxacin exposure only. In addition, the use of enrichments in an *in vitro* experiment allows for high-throughput assessments of complex bacterial communities, which would be more costly and time-consuming in *in vivo* models, making the study design appropriate for initial screening of possible bacterial community responses to contaminant exposure (Hjelset, 2021)

5 Conclusions

High concentrations of leachates from washing gloves significantly and negatively affected the yield and maximum growth rates and prolonged the lag phases of sea urchin microbiome enrichments. The bacterial community composition showed significant responses in alpha diversity estimates in response to increased leachate concentrations. The increased ACE richness and Simpson index indicated possible decreases in the most abundant bacterial genera, being replaced by a higher number of less abundant species. No effects on beta diversity were evident. Significant positive as well as negative log fold changes in individual ASVs of different bacterial genera were observed under leachate exposure. These results constitute a first step into understanding how plastic leachates might affect different heterotrophic bacterial groups. Furthermore, it indicates that leachates inhibit bacterial growth, but also promote complex bacterial community assembly changes.

In addition to adverse effects of leachates, ciprofloxacin exposure on preconditioned bacteria induced significant negative interaction effects on maximum growth rates, indicating possible synergistic effects of leachates and ciprofloxacin. Although bacterial yield and lag phases were significantly affected by both leachates and ciprofloxacin no significant interaction effect was observed. No significant alpha or beta diversity changes were observed resulting from interaction effects of leachate and ciprofloxacin exposure, and only leachates induced changes in beta diversity. Although significant log fold changes in individual ASVs for different bacterial genera were observed, these did not indicate probable adaptations towards ciprofloxacin after leachate exposure.

Overall, this study did not observe responses that indicate an increased ciprofloxacin resistance resulting from preconditioning with leachates. Plastic leachates and ciprofloxacin can negatively affect the enriched microbiome of *Brissopsis lyrifera,* and the combination of the two contaminants may potentiate each other for bacterial growth, leading to increased negative effects on the microbial community functions. A logical next step is mesocosm experiments with benthic animals to study the effects of leachates on marine invertebrate microbiomes in situ, and subsequent effects on the ecosystem services performed by the invertebrates, and thus the potential of leachates to alter marine ecosystem functions.

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Appendix

1. Modified YCFA medium preparation protocol

Microorganisms DSMZ

1611. YCFA MEDIUM (modified)

Casitone	10.00	g
Yeast extract	2.50	g
Glucose	5.00	g
$MgSO_4$ x 7 H_2O	45.00	mg
$CaCl_2$ x 2 H_2O	90.00	mg
K_2HPO_4	0.45	g
KH_2PO_4	0.45	g
NaCl	0.9	g
Resazurin	1.0	mg
Distilled water	1000.00	ml
$NaHCO_3$	4.00	g
L-Cysteine-HCl	1.00	g
Hemin	10.00	mg
Volatile fatty acids:		
Acetic acid	1.90	ml
Propionic acid	0.70	ml
iso-Butyric acid	90.00	µl
n-Valeric acid	100.00	µl
iso-Valeric acid	100.00	µl
Vitamin solution:		
Biotin	2.00	mg
Folic acid	2.00	mg
Pyridoxine-HCl	10.00	mg
Thiamine-HCl x 2 H_2O	5.00	mg
Riboflavin	5.00	mg
Nicotinic acid	5.00	mg
D-Ca-pantothenate	5.00	mg
Vitamin B_{12}	0.10	mg
p-Aminobenzoic acid	5.00	mg
Lipoic acid	5.00	mg
Distilled water	1000.00	ml

Dissolve ingredients except $NaHCO_3$, hemin, cysteine in water and boil for 10 min, then cool to room temperature while gassing with 100% CO_2.Add the $NaHCO_3$, hemin, cysteine and adjust pH to 6.7 -6.8. Distribute under N_2 into Hungate tubes (10 ml) and autoclave. Filter sterilize vitamin solution and aseptically add 100 µl per Hungate tube.

2. The chemicals identified in the plastic leachates

Name	CAS_id	2020-1132_DCM-EtAC	Labblank_DCM-EtAC
Benzenamine, 2,4-dimethyl-	95-68-1	86	
2-Hydroxy-iso-butyrophenone	7473-98-5	91	
2,4,7,9-Tetramethyl-5-decyn-4,7-diol	126-86-3	95	
Butylated Hydroxytoluene	128-37-0	94	
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-, methylcarbamate	02.11.1918	90	
2(3H)-Benzothiazolone	934-34-9	85	
Benzene, 1,1'-(1,3-propanediyl)bis-	1081-75-0	91	
Methanone, (1-hydroxycyclohexyl)phenyl-	947-19-3	85	
Heptadecane	629-78-7	86	
Octane, 2,7-dimethyl-	1072-16-8	85	
1-Propanol, 2-(2-hydroxypropoxy)-	106-62-7	87	
Hexanamide	628-02-4	91	
Nonanamide	1120-07-6	90	
Tetradecanoic acid	544-63-8	85	
,beta,-Phenylpropiophenone	1083-30-3	88	
2-Mercaptobenzothiazole	149-30-4	92	
Nonadecane	629-92-5	91	89
Undecane, 4,7-dimethyl-	17301-32-5	93	
Phthalic acid, 6-ethyl-3-octyl butyl ester	1000315-17-4	89	
Nonanamide	1120-07-6	88	
n-Hexadecanoic acid	57-10-3	94	
Norbornane, 2-isobutyl-	18127-14-5	88	
1-Hexadecanol, acetate	629-70-9	95	
1-Undecanol, acetate	1731-81-3	87	
Acetic acid, decyl ester	112-17-4	87	
Pentadecanal-	09.11.2765	96	
1-Octadecanol	112-92-5	97	
9,12-Octadecadienoic acid (Z,Z)-	60-33-3	94	
9(E),11(E)-Conjugated linoleic acid	544-71-8	95	
9-Octadecenoic acid, (E)-	112-79-8	92	
Hexadecanamide	629-54-9	93	
Octadecanoic acid	57-11-4	95	
11-cis-Vaccenyl acetate	6186-98-7	91	
Z-13-Octadecen-1-yl acetate	60037-58-3	90	
Acetic acid n-octadecyl ester	822-23-1	99	
9-Octadecenamide, (Z)-	301-02-0	87	
Octadecanamide	124-26-5	91	
Eicosen-1-ol, cis-9-	112248-30-3	86	

4H-Cyclopentacyclooctene, decahydro-	6663-95-2	90	
Eicosyl acetate	822-24-2	97	
1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	74746-55-7	89	
Zinc, bis(diethylcarbamodithioato-S,S')-, (T-4)-	14324-55-1	94	
Squalene	111-02-4	94	92
(R)-2,7,8-Trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)chroman-6-ol	14101-61-2	88	
2H-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-	1721-51-3	91	
Stigmasterol	83-48-7	86	
,gamma,-Sitosterol	83-47-6	96	
Stigmasta-5,24(28)-dien-3-ol, (3,beta,,24Z)-	481-14-1	94	
Zinc dibutyldithiocarbamate	136-23-2	93	

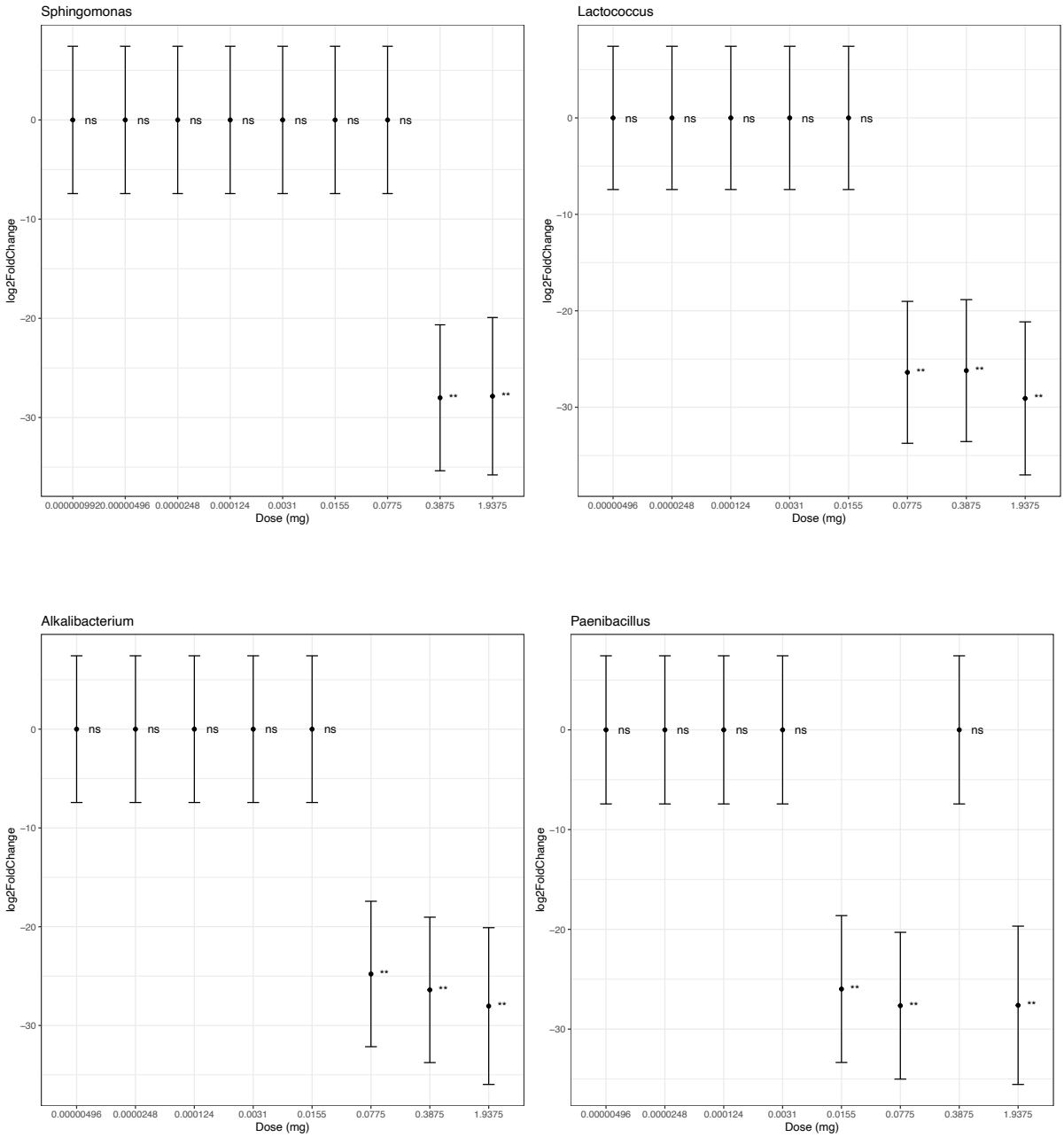
3. Content in all samples in the leachate exposure experiment

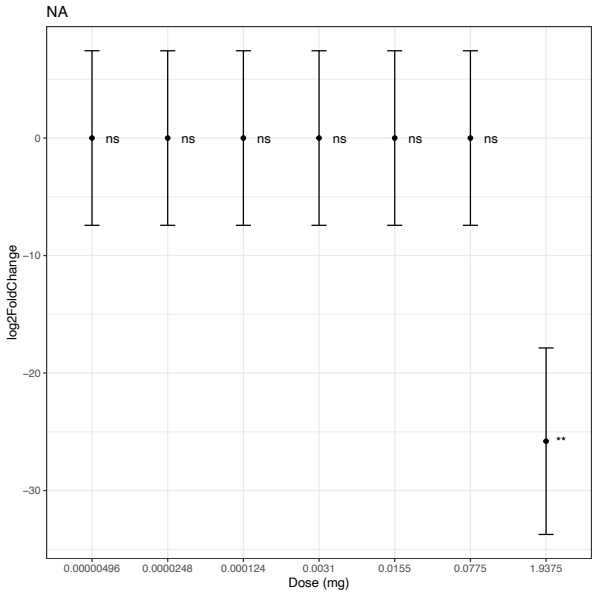
Treatment ID	**Final concentration of leachates (mg/mL)**	**Final concentration of 3,5 DCP (mg/mL)**	**Bacteria (µl)**	**DMSO (µl)**	**Medium (µl)**	**Leachates (µl)**
C1	1.9375	0	1	0	194	5
C2	0.3875	0	1	0	194	5
C3	0.0775	0	1	0	194	5
C4	0.0155	0	1	0	194	5
C5	0.0031	0	1	0	194	5
C6	0.00062	0	1	0	194	5
C7	0.000124	0	1	0	194	5
C8	0.0000248	0	1	0	194	5
C9	0.00000496	0	1	0	194	5
C10	0.000000992	0	1	0	194	5
C0	0	0	1	0	199	0
DMSO	0	0	1	6	194	0
Medium	0	0	1	0	199	0
POS	0	0.0066	1	0	194	0
Blank	0	0	0	0	200	0

4. Content in all samples in the ciprofloxacin exposure experiment

Leachate ID	Ciprofloxacin ID	Final leachates concentration (mg/mL)	Final Ciprofloxacin concentration (mg/mL)	Bacteria (µl)	Medium (µl)	Ciprofloxacin (µl)
C0	**A0**	0	0	1	199	0
C0	**A1**	0	0.000000025	1	194	5
C0	**A2**	0	0.0000025	1	194	5
C0	**A3**	0	0.00025	1	194	5
C8	**A0**	0.0000248	0	1	199	0
C8	**A1**	0.0000248	0.000000025	1	194	5
C8	**A2**	0.0000248	0.0000025	1	194	5
C8	**A3**	0.0000248	0.00025	1	194	5
C5	**A0**	0.0031	0	1	199	0
C5	**A1**	0.0031	0.000000025	1	194	5
C5	**A2**	0.0031	0.0000025	1	194	5
C5	**A3**	0.0031	0.00025	1	194	5
C3	**A0**	0.0775	0	1	194	0
C3	**A1**	0.0775	0.000000025	1	194	5
C3	**A2**	0.0775	0.0000025	1	194	5
C3	**A3**	0.0775	0.00025		194	5
POS	**A0**	0	0	1	194	0
POS	**A1**	0	0.000000025	1	194	5
POS	**A2**	0	0.0000025	1	194	
POS	**A3**	0	0.00025	1	194	
Blank	**Blank**	0	0	0	200	0
C0	**ABCTRL**	0	0.25	1	194	5

5. ASVs from bacterial genera with significant log fold changes in response to leachates exposure

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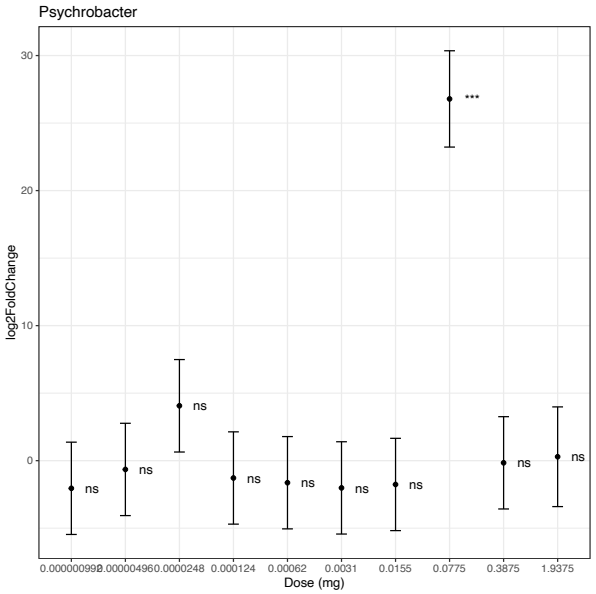
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Dose (mg)
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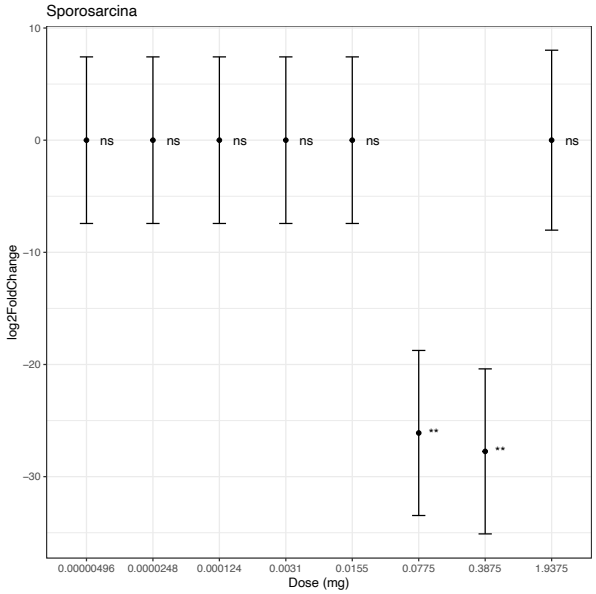
0.3875
1.9375
***
ns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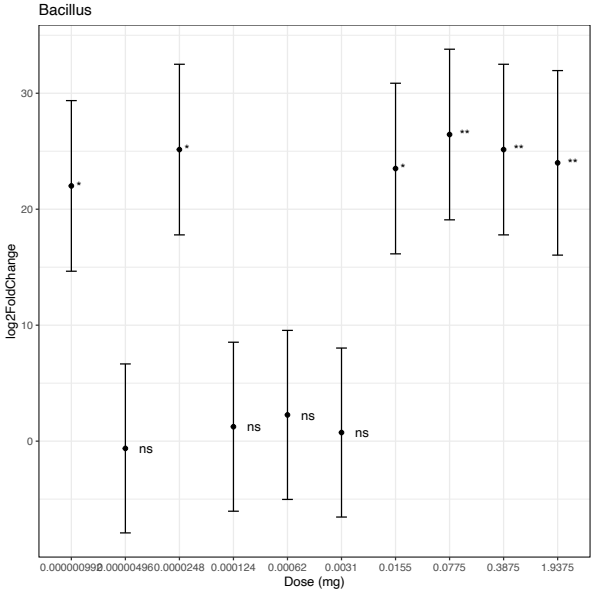
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Dose (mg)
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0.3875
1.9375
*
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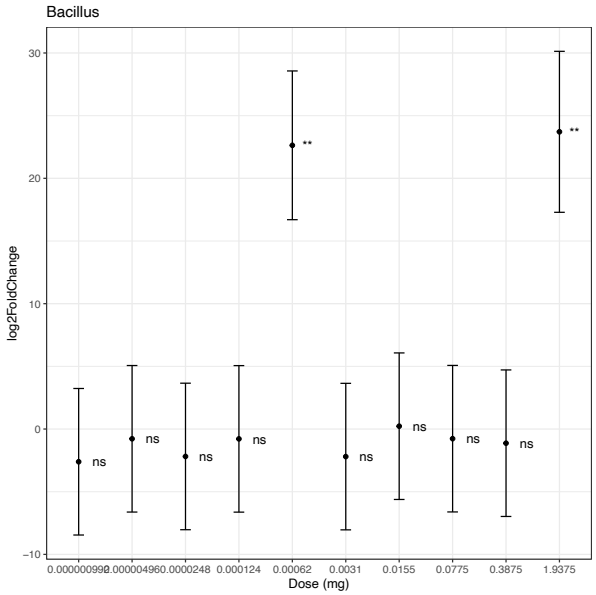
ns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1.9375
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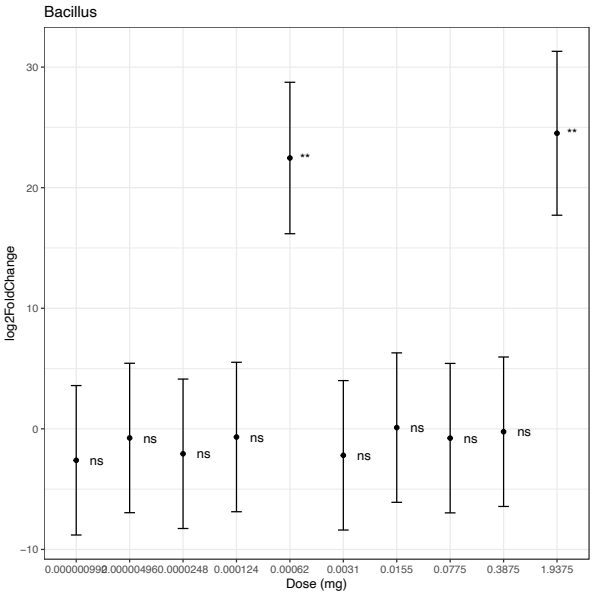
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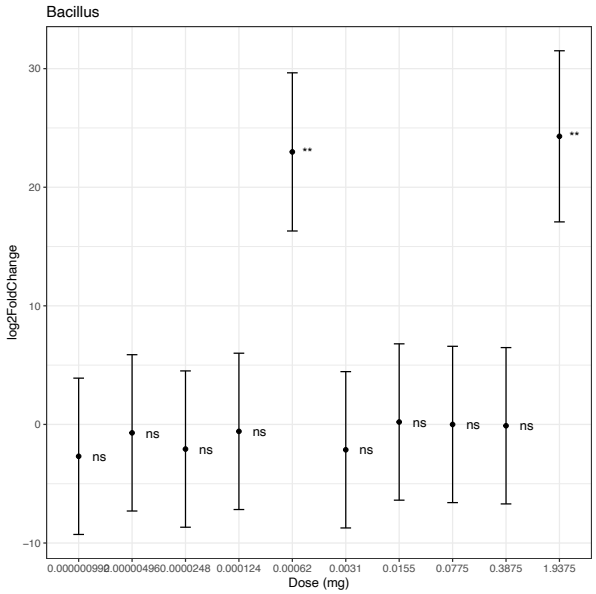
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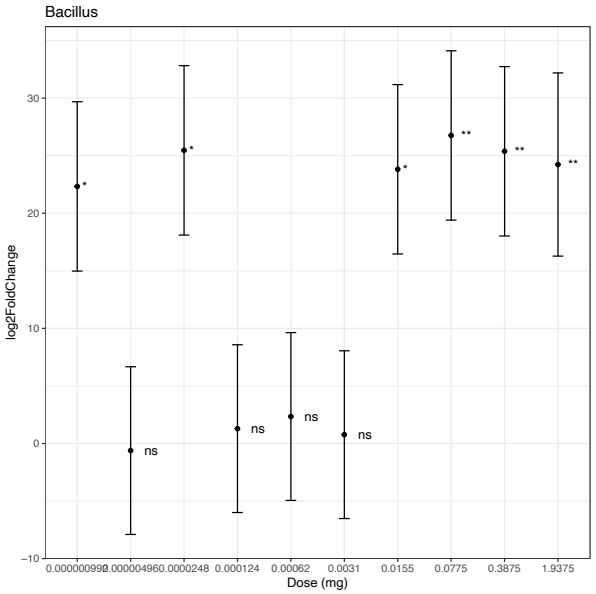
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Dose (mg)
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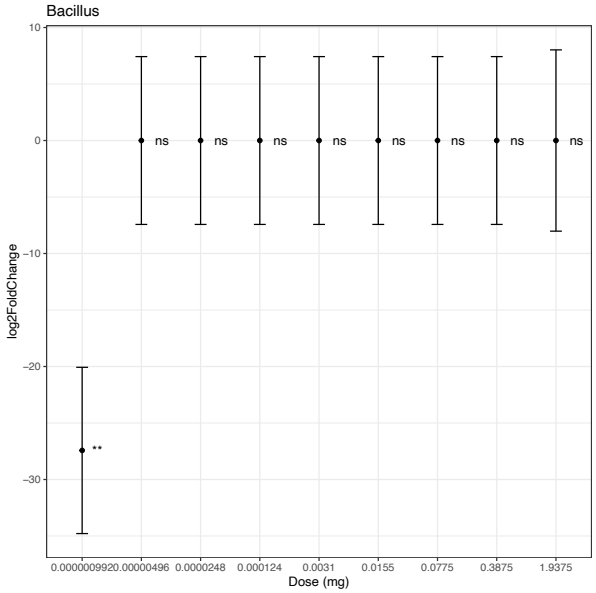
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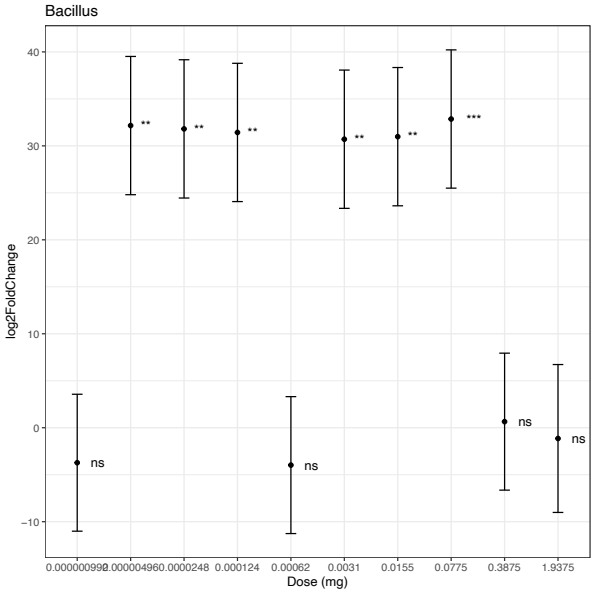
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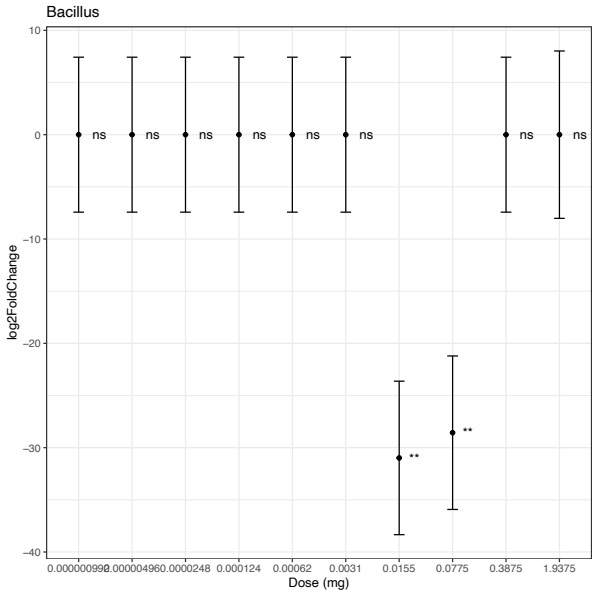
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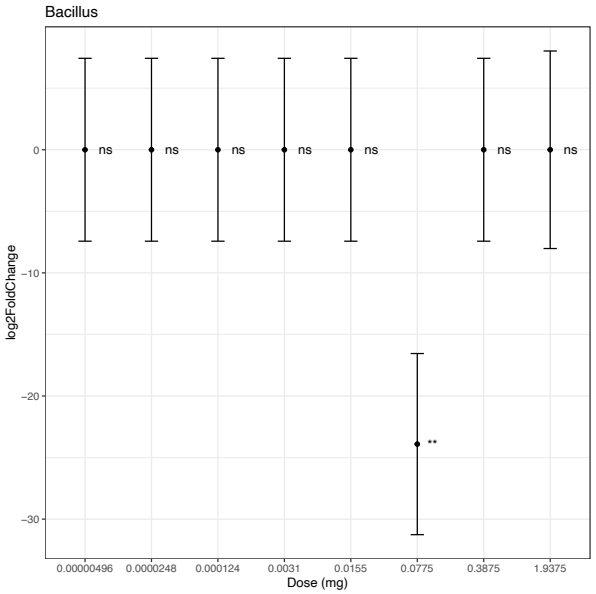
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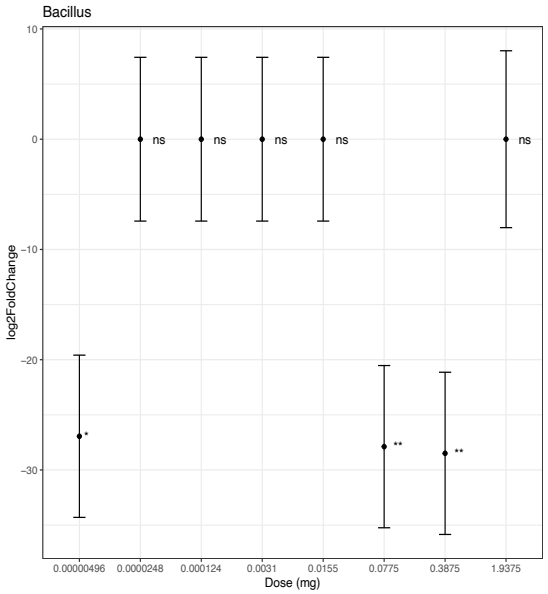
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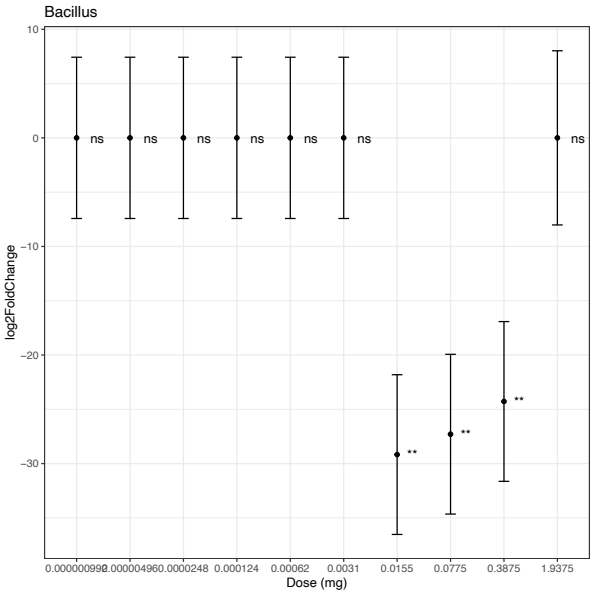
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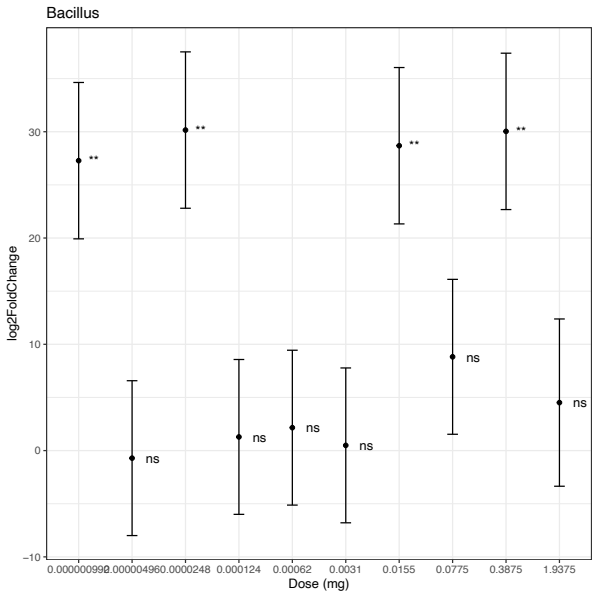
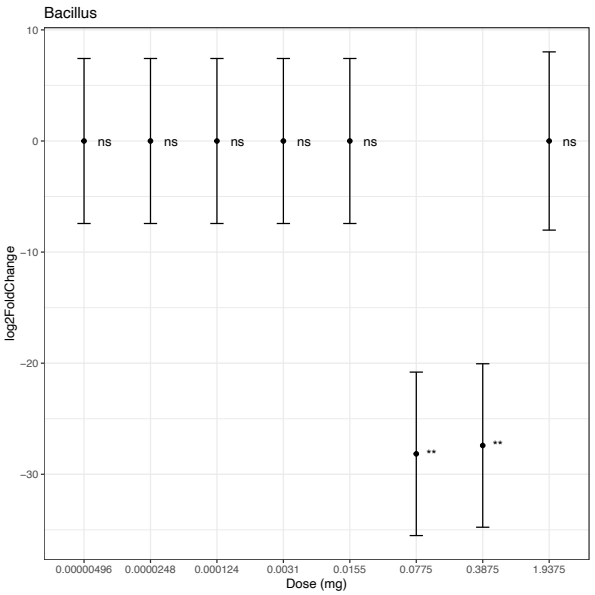
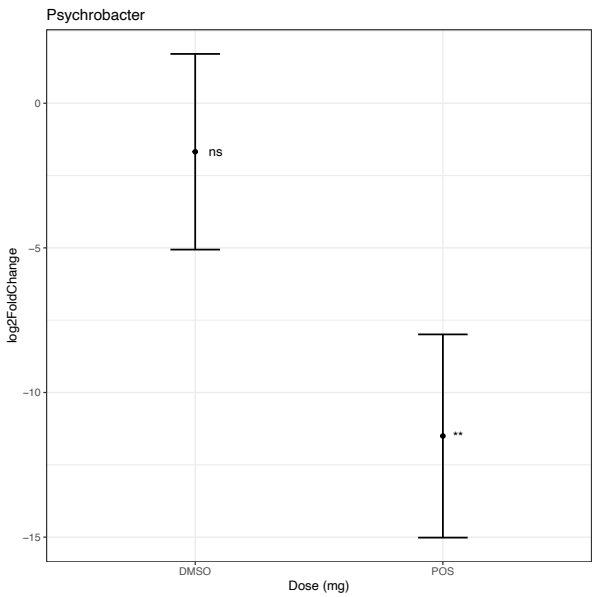
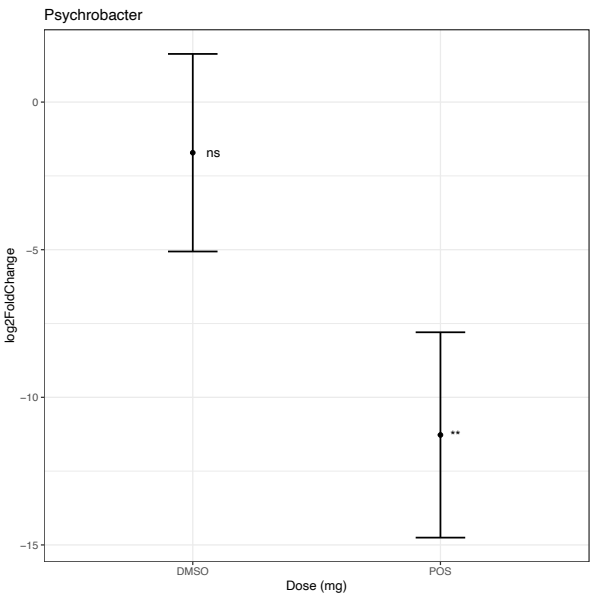
0.0775
0.3875
1.9375
ns
**

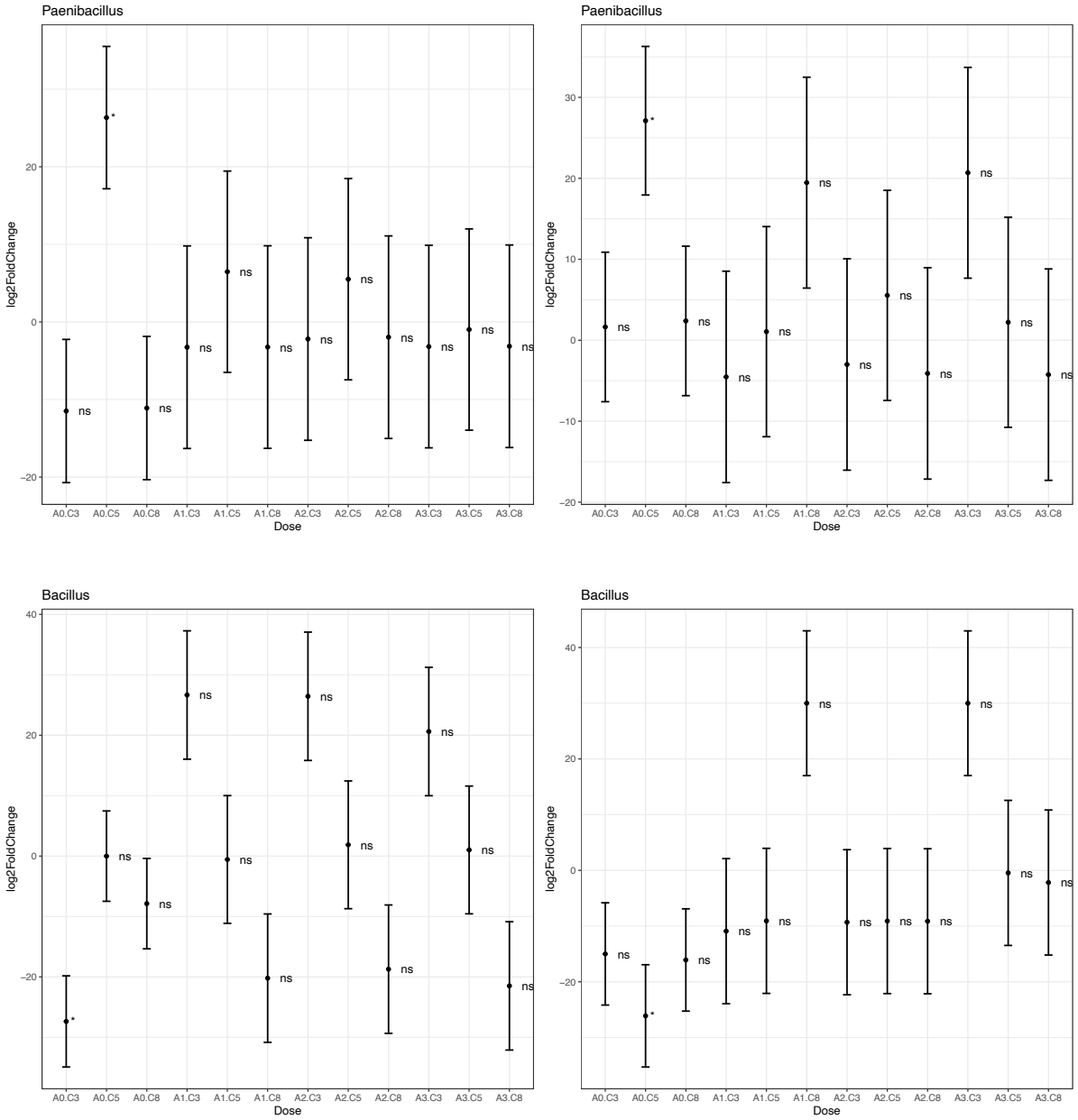

Bacillus
log2FoldChange
Dose (mg)
0.00000992
0.00000496
0.0000248
0.000124
0.00062
0.0031
0.0155
0.0775
0.3875
1.9375
**
ns

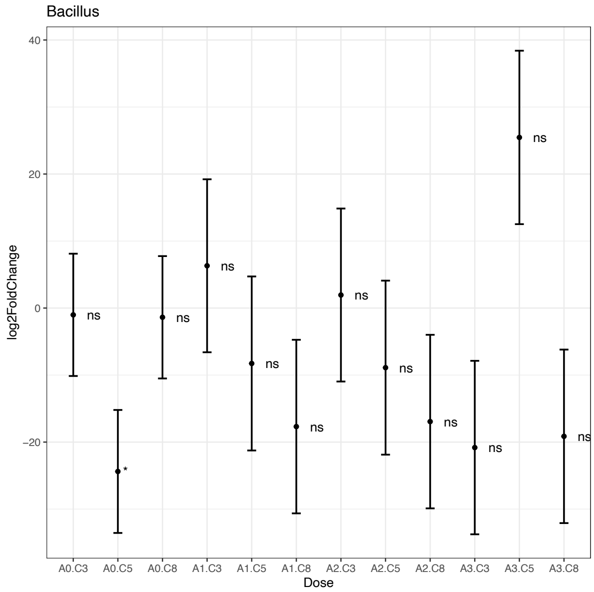

Bacillus
log2FoldChange
Dose (mg)
0.00000496
0.0000248
0.000124
0.0031
0.0155
0.0775
0.3875
1.9375
ns
**

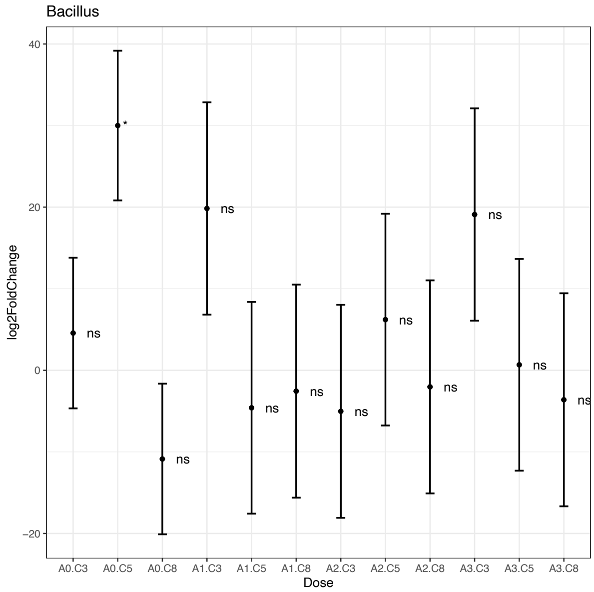

Psychrobacter
log2FoldChange
Dose (mg)
DMSO
POS
ns
**

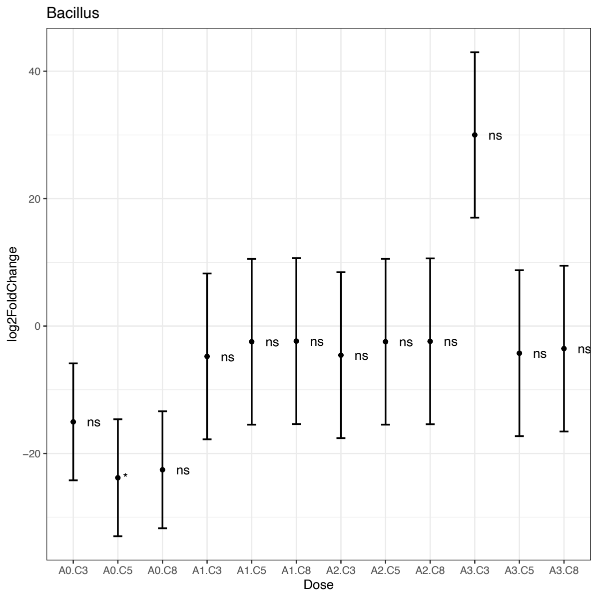

Psychrobacter
log2FoldChange
Dose (mg)
DMSO
POS
ns
**

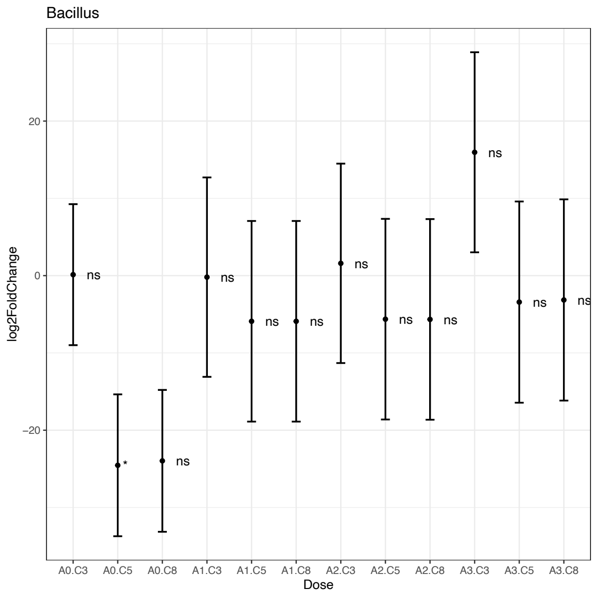
6. ASVs from bacterial genera with significant log fold changes in response to ciprofloxacin exposure on preconditioned enrichments

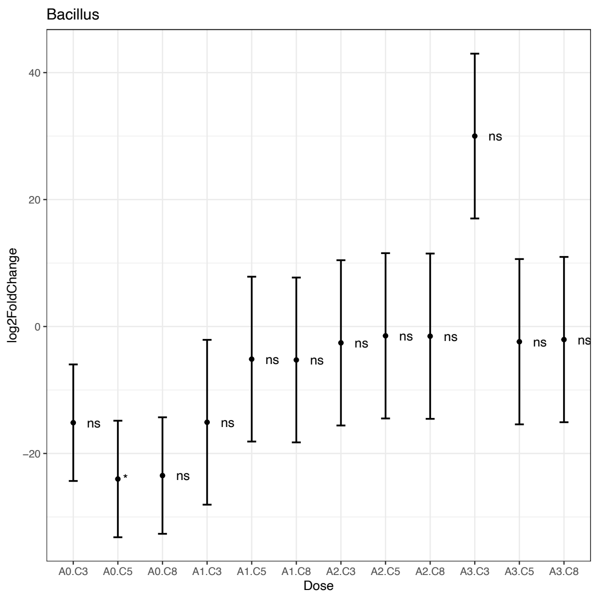


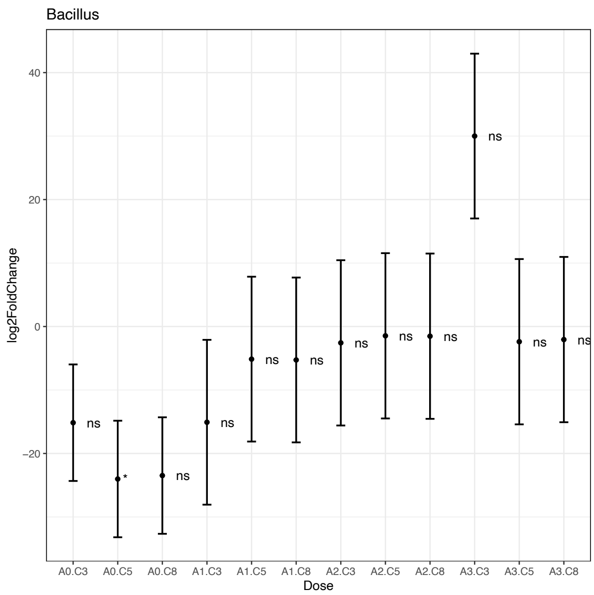

Bacillus
40
20
0
−20
log2FoldChange
A0.C3 A0.C5 A0.C8 A1.C3 A1.C5 A1.C8 A2.C3 A2.C5 A2.C8 A3.C3 A3.C5 A3.C8
Dose
ns
*

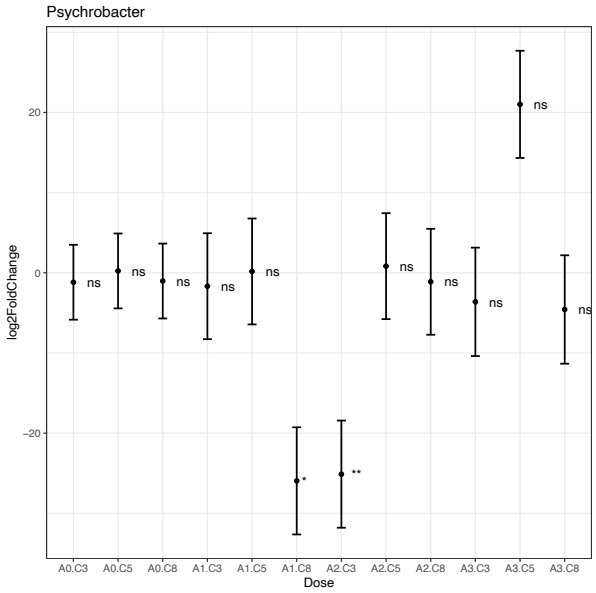

Bacillus
40
20
0
−20
log2FoldChange
A0.C3 A0.C5 A0.C8 A1.C3 A1.C5 A1.C8 A2.C3 A2.C5 A2.C8 A3.C3 A3.C5 A3.C8
Dose
ns
*

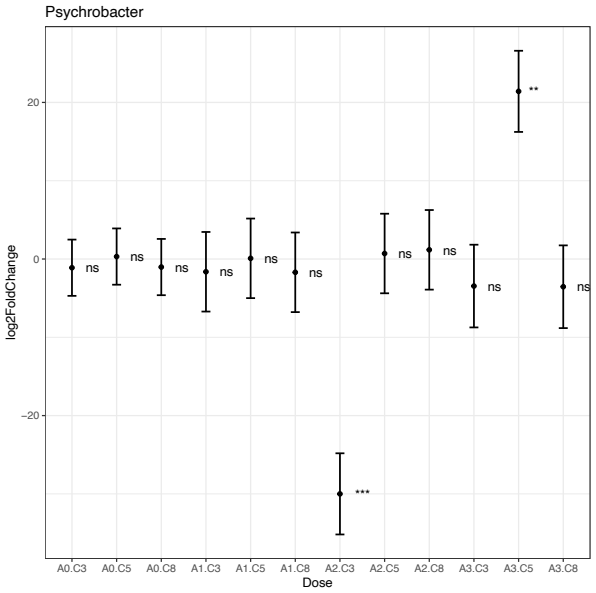

Bacillus
40
20
0
−20
log2FoldChange
A0.C3 A0.C5 A0.C8 A1.C3 A1.C5 A1.C8 A2.C3 A2.C5 A2.C8 A3.C3 A3.C5 A3.C8
Dose
ns
*


Bacillus
20
0
−20
log2FoldChange
A0.C3 A0.C5 A0.C8 A1.C3 A1.C5 A1.C8 A2.C3 A2.C5 A2.C8 A3.C3 A3.C5 A3.C8
Dose
ns
*


Bacillus
40
20
0
−20
log2FoldChange
A0.C3 A0.C5 A0.C8 A1.C3 A1.C5 A1.C8 A2.C3 A2.C5 A2.C8 A3.C3 A3.C5 A3.C8
Dose
ns
*


Bacillus
40
20
0
−20
log2FoldChange
A0.C3 A0.C5 A0.C8 A1.C3 A1.C5 A1.C8 A2.C3 A2.C5 A2.C8 A3.C3 A3.C5 A3.C8
Dose
ns
*


Psychrobacter
log2FoldChange
20
0
−20
ns
ns
ns
ns
ns
*
**
ns
ns
ns
ns
ns
A0.C3
A0.C5
A0.C8
A1.C3
A1.C5
A1.C8
A2.C3
A2.C5
A2.C8
A3.C3
A3.C5
A3.C8
Dose


Psychrobacter
log2FoldChange
20
0
−20
ns
ns
ns
ns
ns
ns
***
ns
ns
ns
**
ns
A0.C3
A0.C5
A0.C8
A1.C3
A1.C5
A1.C8
A2.C3
A2.C5
A2.C8
A3.C3
A3.C5
A3.C8
Dose