The effects of a synthetic and biological surfactant on the community composition and metabolic activity of a freshwater biofilm

Running Title: Surfactant effects: Biofilm composition and metabolism

Authors: Stephanie P. Gill[1], William R. Hunter[2], Laura Coulson[3,4], and Jakob Schelker[3,5]

[1]Department of Geography and Environmental Studies, Ulster University Coleraine, Northern Ireland

[2]Fisheries and Aquatic Ecosystems Branch, Agri-Food and Biosciences Institute, Belfast, Northern Ireland

[3]WasserCluster Lunz, Lunz Am See, Austria

[4]Institute of Hydrobiology and Aquatic Ecosystem Management, University of Natural Resources and Life Sciences, Vienna, Austria

[5] Department of Functional and Evolutionary Ecology, University of Vienna, Vienna, Austria

*Correspondence: Stephanie P. Gill

School of Geography and Environmental Science
Ulster University, Coleraine Campus
BT52 1SA. United Kingdom
Email: gill-S5@ulster.ac.uk
Telephone: 07541-036849

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Originality-Significance Statement

To our knowledge this is the first study that examines the effects of the chemical surfactant sodium dodecyl sulphate and the biological surfactant rhamnolipid on a mixed species freshwater biofilm. Research typically focuses on single species biofilms in medical or industrial settings. Therefore, we are also the first study that examines the community composition and metabolic activity of a mixed freshwater biofilm exposed to these surfactants, and are the only study that has examined the carbon and nitrogen content of an exposed aquatic biofilm. Our results will help to shape policy surrounding the input of contaminants of emerging concern such as surfactants into waterways, and potentially to help manage the use of these chemicals within industrial settings as a means to control biofilms.

Summary

Surfactants are used to control microbial biofilms in industrial and medical settings. Their known toxicity on aquatic biota and their longevity in the environment has encouraged research on biodegradable alternatives such as rhamnolipids. While previous research has investigated the effects of biological surfactants on single species biofilms, there remains a lack of information regarding the effects of synthetic and biological surfactants in freshwater ecosystems. We conducted a mesocosm experiment to test how the surfactant sodium dodecyl sulphate (SDS) and the biological surfactant rhamnolipid altered community composition and metabolic activity of freshwater biofilms. Biofilms were cultured in the flumes using lake water from Lake Lunz in Austria, under high (300 ppm) and low (150 ppm) concentrations of either surfactant over a four week period. Our results show that both surfactants significantly affected microbial diversity. Up to 36% of microbial OTUs were lost after surfactant exposure. Rhamnolipid exposure also increased the production of the extracellular enzymes leucine aminopeptidase and glucosidase, while SDS exposure reduced leucine aminopeptidase and glucosidase. This study demonstrates that exposure of freshwater biofilms to chemical and biological surfactants caused a reduction of microbial diversity and changes in biofilm metabolism, exemplified by shifts in extracellular enzyme activities.

Introduction

In aquatic ecosystems, microorganisms live preferentially in microbial biofilms and act as major drivers of the biogeochemical cycling of carbon, nitrogen, and other nutrients (Paul *et al.*, 1991; Decho, 2000; Dang and Lovell, 2016; Flemming *et al.*, 2016; Battin *et al.*, 2016). In streams and rivers, microbial biofilms play an important role in the carbon cycle (Lyon and Ziegler, 2009; Qu *et al.*, 2017). Microorganisms within biofilms are able to break down dissolved organic matter and utilize the dissolved organic carbon (DOC) within the compounds (Romani *et al.*, 2003). Through mineralization, aquatic biofilms are then able to produce large quantities of carbon dioxide for release back into the atmosphere (Kroukamp and Wolfaardt, 2008). Similarly, depending on the nitrogen levels in the system, microbial biofilms resident in fluvial sediments can substantially reduce inorganic nitrogen (N) loads in N-rich aquatic ecosystems through denitrification (Zhang *et al.*, 2016; Cui *et al.*, 2017). In N-limited aquatic systems, they may help to facilitate the transfer of N to higher trophic levels (Dodds *et al.*, 2000).

While microbial biofilms play an important ecological role, they are also of concern in industrial and medical applications. For example, biofilms can facilitate the growth of antibiotic resistant bacteria (Lindsay and von Holy, 2006; Srey *et al.*, 2013; Bowler *et al.*, 2020). Synthetic surfactants, such as sodium dodecyl sulphate (SDS), are widely used in personal care and cleaning products. They reduce biofilm growth in a wide range of industrial, medical, and environmental contexts (Waaler *et al.*, 1993; Landa *et al.*, 1999; Rigotti *et al.*, 2017; Sloup *et al.*, 2016; Ueda *et al.*, 2019). Surfactants are amphiphilic chemicals that reduce surface tension within a liquid through the formation of micelles. Micelles occur when molecules form an aggregate with hydrophobic tails on the inside (Schmitz, 2018) and hydrophilic heads on the outside of an amphiphilic compound (Milovanovic *et al.*, 2017). An early study estimated the

global use of surfactants at 36.4 million tons per year (Hauthal, 2004; Mungray and Kumar, 2009), suggesting that synthetic surfactant may be commonly transferred into freshwater ecosystems. A previous report by the European Commission (Thornton *et al.*, 2001) has highlighted surfactants as one of the top contaminants found in wastewater treatment effluent. Given the mass of surfactants produced every year, it can well be anticipated that surfactants still represent some of the most abundant chemicals in municipal and industrial wastewater outflow.

Wastewater treatment plants (WWTPs) have been observed to have very high levels of surfactants in their inflows (for example, up to 1900 ug L^{-1} for LAS and 40,000 ug L^{-1} for alkyl ethoxysulfates (AES) (Gomez *et al.*, 2011)) with removal rates of only 40-90% during waste-water treatment (Gomez *et al.*, 2011; Kruszelnicka *et al.*, 2019). Similarly, surfactant levels of raw water intakes of drinking water treatment plants (DWTPs) can be high (up to 49 ug L^{-1} for LAS and 114 ug L^{-1} for AES) (Gomez *et al.*, 2011) with only partial removal of surfactants by DWTPs. Finally, high levels of surfactants have also been identified in surface sediments obtained near wastewater treatment plant discharge areas into coastal waters, such as the Jamaica Bay of New York (Lara-Martín *et al.*, 2012). This underlines the presence and persistence of surfactants in aquatic ecosystems downstream of urban and industrial environments.

Research on the effects of surfactants on microbial biofilms has primarily focused on single-species environmental biofilms or those commonly found in medical and industrial environments. Direct applications of the anionic synthetic surfactant SDS has shown significant disruption of growth and development of *Pseudomonas aeruginosa* biofilms (Díaz De Rienzo *et al.*, 2016; Nguyen *et al.*, 2020). Exposure to SDS has also decreased biofilm density and flattened its shape (Robinson *et al.*, 2006). Effects on other single species or medical biofilms have also been investigated including methicillin-resistant *Staphylococcus aureus* (Ueda *et al.*,

2019), *Pseudomonas fluorescens* (Simoes *et al.,* 2008), and *Salmonella enterica* serovar *typhimurium* (Mireles II *et al.,* 2001). Inhibited biofilm formation was seen to occur within all investigations.

The use of synthetic surfactants has been questioned due to the potential environmental impact, specifically the longevity and toxicity to aquatic biota. For example, previous research has shown that synthetic surfactants, such as Triton X-100 (Octylphenol Ethoxylate), are not easily biodegradable under aerobic nor anaerobic conditions (Mohan *et al.,* 2006). Other synthetic surfactants such as SDS have also been noted as toxic to a variety of microorganisms such as *Vibrio fischeri,* fish and shellfish such as *Clarias batrachus* and *Mytilus galloprovincialis,* yeast, and also small invertebrates, such as *Daphnia magna,* and *Tigriopus fulvus* (Mariani *et al.,* 2006; Lima *et al.,* 2011; Franzetti *et al.,* 2012; Messina *et al.,* 2014; Cao *et al.,* 2020; Kumar *et al.,* 2020). Research has therefore shifted towards identifying a natural alternative that may be less harmful to the aquatic environment.

Biofilm-dwelling microorganisms naturally produce a number of surfactants to control the surrounding extracellular polymeric substance (EPS) matrix. The most common substances of this group are rhamnolipids and sophorolipids which are glycolipids formed by the combination of either a long-chain aliphatic acid or hydroxyaliphatic acid with a carbohydrate (Desai and Banat, 1997). When exposing single-cell biofilms of *P. aeruginosa* to rhamnolipids, growth and development were disrupted, and cell viability was reduced (De Rienzo *et al.,* 2015). Disruptions by rhamnolipids have also been shown for single species biofilms of *Staphylococcus aureus* (Quinn *et al.,* 2013; Aleksic *et al.,* 2017; Tambone *et al.,* 2021), *Bacillus pumilus* (Dusane *et al.,* 2010), *and Rhodococcus erythropolis* (Schreiberova *et al.,* 2012). Yet, rhamnolipids are biodegradable in a variety of environments, including under aerobic and

anaerobic conditions (Mohan *et al.*, 2006; Ramos da Silva *et al.*, 2019). Biological surfactants can also function in more varied environmental conditions such as large pH and temperature ranges (Muthusamy *et al.*, 2008). Given that previous research primarily addressed the effects of biological surfactants on single species biofilms of bacteria relevant to medical settings, there remains a knowledge gap on how the exposure of multi-species freshwater biofilms to surfactants affects biofilm microbial community composition and function.

There is a growing body of evidence that anthropogenic trace contaminants such as pharmaceuticals, herbicides, and antimicrobial agents can have major impacts upon the composition and functioning of aquatic microbial communities (Rosi-Marshall *et al.*, 2013; Harjung *et al.*, 2020; McClean and Hunter, 2020; Ke *et al.*, 2020; Kusi *et al.*, 2020). Exposure to contaminants such as the antibacterial agent Tricolsan (Hay *et al.*, 2001; Drury *et al.*, 2013) and the herbicide Diuron (Sumpono *et al.*, 2003; Seghers *et al.*, 2003) have been observed to place microbial communities under a selective pressure that favors microorganisms that can use these contaminants as an additional carbon source. Given that levels of surfactants and their metabolites are consistently among the top contaminants identified within wastewater influent and groundwater (Lara-Martín *et al.*, 2014; Schaider *et al.*, 2014), it is reasonable to infer that they could represent a major control upon the microbial communities within human affected inland waters.

This study investigated the effects of the synthetic surfactant SDS and the biological surfactant rhamnolipid on microbial biofilm community composition, microbial diversity, and metabolic activity. Using recirculating flume mesocosms, we aimed to answer the following overarching scientific question: What are the effects of a synthetic and biological surfactant on a natural aquatic microbial biofilm, and how do these effects compare? We hypothesized that

biofilm exposure to a surfactant will decrease bacterial biodiversity, as the surfactants impact microbial community composition and causes the loss of sensitive bacterial taxa. Secondly, we hypothesize that the changes in microbial community composition will alter biofilm metabolic activity. The loss of sensitive taxa will allow generalists to dominate who do not have the same primary functions and efficiency as more specialized taxa, reducing biofilm performance, expressed by metabolic activity. From this, we predict that the concentrations of extracellular enzymes produced by the biofilms, used as a proxy for community-scale metabolic activity within the biofilms, will be lower in the surfactant treatments. We tested these hypotheses by quantifying changes in community composition through 16s rRNA analysis and changes in biofilm structure and metabolic activity through different analytical methods, such as changes in gross primary productivity (GPP), community respiration (CR), ash free dry mass (AFDM), Carbon: Nitrogen ratios (C:N), and extracellular enzyme activity (EEA).

Results

Primary productivity and community respiration

We found no significant differences in primary productivity (ANOVA, F=0.022, p=0.978) and community respiration (ANOVA, F=0.148, p=0.865) of the treatments versus the controls (Figures 1A, 1B). All treatments showed substantial variation among replicates (Figure 1), with the most pronounced variability being present in respiration. Coefficients of variation were highest for respiration in the controls (1.56, see Table 1) and lowest (0.021) in the rhamnolipid 150 treatment. Rhamnolipid treated systems overall had the lowest variation for CR, while the controls had the highest.

Ash free dry mass and C:N ratios

We found no significant difference among treatments and controls (ANOVA F=0.045, p=0.957) for AFDM. The coefficients of variation, however, showed differences in variation among the treatments (Table 1), with higher surfactant concentrations decreasing variation (e.g., rhamno 150 has greater variation than rhamno 300) (Figure 1C). Control variation was comparable to both low dose surfactant variations. However, C (ANOVA F=18.36, p=0.0028) and N (ANOVA F=5.471, p=0.044) content was significantly different by mass among treatments (Figure 2A). Differences lie between the rhamno 300 and the control for both C (Tukey HSD p=0.0063) and N (Tukey HSD p=0.041) content, and between SDS 300 and the control for C (Tukey HSD p=0.0036) content.

We found no significant differences among treatments and controls for C:N ratios by mass (Kruskal-Wallis p=0.193) present in the biofilms (Figure 2B). There were differences in variation among treatments and controls (Table 1). Rhamnolipid treated flumes had lower variation than either SDS treated flumes or the controls, however within each treatment a higher surfactant concentration increased variation.

Bacterial community composition

Shannon-Wiener Diversity was significantly different among treatments (ANOVA F=71.702, p<0.0001) with a higher diversity in the control flumes, second highest in the rhamnolipid flumes, and lowest in the SDS flumes, while for Chao1 the differences were not significant (ANOVA F=3.7748, p=0.087). Shannon diversity showed significant differences among all three treatments tested (Tukey HSD rhamnolipid and control; p=0.043, SDS and control; p<0.0001, SDS and rhamnolipid; p<0.001). Significance was driven by surfactant

presence and type, but not concentration. The control had the greatest bacterial diversity, while both rhamnolipid treatments had the next highest, and the lowest belonged to both SDS treatments (Figure 3A).

By testing beta diversity and community differences on the rarefied data, we identified distinct groupings between all treatments and controls, as evident by the NMDS (Figure 3B). The ANOSIM results confirmed the differences among both surfactants and the controls (R=0.9112, p=0.001). Community differences were further identified by looking at only the taxa in the top 80% of all taxa present across all treatments, which was 18 taxa. We identified several genera that were heavily dominant in rhamnolipid-treated flumes. These were: *Brevundimonas spp*., OTU005, *Pseudomonas,* and *Arthrobacter*. In SDS flumes we observed *Brevundimonas terrae, Flavobacterium spp. A*, and in the control flumes *Exiguobacterium, Arenimonas,* and *Stenotrophomona*. We also identified 32 genera only present in the controls, 5 there were exclusive to rhamno 150, 6 in rhamno 300, 19 in SDS 150, and 7 in SDS 300.

Total OTUs within each treatment and OTUs within the top 80% of all taxa per treatment were also calculated (Table 2). The control flumes had the greatest number of OTUs present, and the greatest number of OTUs within their top 80% of taxa. The SDS and rhamnolipid flumes had similar numbers of total OTUs present, but the SDS flumes had 80% of their total OTUs within half the amount of taxa as the rhamnolipids. This indicated a large difference in evenness between treatments, while abundance was fairly similar.

Extracellular Enzyme Activity

According to the DRA, EEA differed among all treatments and controls, with the exception of the rhamnolipid treatments. The differences in DRA-distances were mainly caused

by the differences in EEA of leucine aminopeptidase and phosphatase, although the results were not significant (ANOSIM F=2.9907, p=0.058). The effects of rhamno 300 and SDS 300 on leucine aminopeptidase were significantly different (ANOVA F=5.914 p=0.038, Tukey HSD p=0.032). The addition of rhamnolipid yielded a higher amount of the extracellular enzymes when compared to the control and the addition of SDS reduced production. Rhamnolipid additions also yielded a higher production of glucosidase, while SDS additions reduced production (ANOVA F=5.741 p=0.0404, Tukey HSD p=0.034). While phosphatase did not have any significant differences among treatments (ANOVA F=2.934 p=0.129), with the exception of SDS 150 addition of any surfactant decreases the production of phosphatase when compared to the control (Figure 4).

Discussion

Our experiment showed that exposure to biological and synthetic surfactants alters the metabolic activity and the community composition of a mixed-species freshwater biofilm. The effects of SDS were more pronounced than the effects of rhamnolipid. Our experiment is the first study of our knowledge that examines the effects of these two surfactants on a multi-species near-natural freshwater biofilm, which contrasts with the commonly performed single-species studies. This study was also novel as it directly compared the effects of a synthetic and a biological surfactant and explicitly studied for the first time the C:N ratios and C and N contents of biofilms that are exposed to surfactants.

Primary Productivity, Respiration, Ash Free Dry Mass, and C:N ratios

Chemical exposure decreased biofilm respiratory activity in previous experiments, including surfactant exposure (Simoes *et al.*, 2008) and pharmaceutical exposure (Rosi-Marshall

et al., 2013). Biofilm volume has also been reduced after a single surfactant exposure event, with decreases of 26.5-98.1% occurring between 22-48 hours after exposure (Mireles II *et al.*, 2001; Quinn *et al.*, 2013). However, previous work on surfactant effects have also shown that biofilms demonstrate resistance and can recover full respiratory activity 12 hours after the initial exposure (Simoes *et al.*, 2008) and biomass 5 hours after initial exposure (Nguyen *et al.*, 2020). Therefore, the length of our experiment of 32 days may have been long enough where GPP, CR, and AFDM could be stable over time, despite the permanent exposure.

Altered C:N ratios are indicative of changes in microbial productivity (where reduced C:N ratios may yield greater productivity) (Li *et al.*, 2016) and biofilm formation (where lower C:N ratios increase biofilm volume) (Thompson *et al.*, 2006). As we did not have any alterations in C:N ratios, and no changes in productivity (GPP and CR) or growth (AFDM) among any treatments or controls, our results support these studies. The addition of surfactants provided an added source of C, however as there were consistent C:N ratios across all flumes it can be assumed that our system was C limited. It is also likely that a thickened EPS matrix, which may be used as a defense mechanism in biofilms in response to surfactant exposure, could be another source of extra carbon (Jefferson, 2004; Zakaria and Dhar, 2020). In contrast, in systems where C is not limiting, an increase in C inputs through surfactants may increase C:N ratios, and therefore negatively affect biofilm productivity and growth.

Diversity and bacterial community composition

Our controls had taxa present that were minimally identified within the surfactant flumes. These taxa included species (spp.) of *Exiguobacterium*, *Acinetobacter*, *Arenimonas*, and *Stenotrophomonas*. The addition of either surfactant created an environment unsuitable for these

bacteria, demonstrating how surfactant exposure can alter biofilm community composition. The control flumes had the highest amount of OTUs present (*n*=203) and the highest diversity, which indicates that both the diversity and abundance of bacteria present in the biofilms decreases after surfactant exposure.

Rhamnolipids are studied for their ability to reduce pre-existing biofilms and prevent future biofilm growth with some success (Quinn *et al.,* 2013; Dusane *et al.,* 2010; De Rienzo *et al.,* 2015). Current research has also shown that rhamnolipid exposure on soil bacteria can shift community composition, although many studies do not examine differences to the genus level (Lu *et al.,* 2019; Wei *et al.,* 2020; Akbari *et al.,* 2021). Our study showed that, much like soil communities, aquatic biofilms experience significant community changes after exposure to rhamnolipids. Further examination into the bacterial genera indicate large increases in a *Brevundimonas* spp., *Pseudomonas* spp., *Arthrobacter* spp., and OTU005 when compared to all other treatments and to the controls. One OTU of *Arthrobacter* spp. was only present within the rhamnolipid flumes, with the exception of one control flume that had low levels of that particular OTU.

SDS caused the largest reduction in microbial diversity when compared with the other treatments and controls. SDS has been researched and used for its ability to degrade biofilms in applications where biofilms are undesirable (Izano *et al.,* 2007; Ueda *et al.,* 2019; Nguyen *et al.,* 2020). This is particularly due to their ability to bind to hydrophobic protein components, introducing a negative charge to the structure which unfolds the bound protein (Gudiksen *et al.,* 2006; Fong and Yildiz, 2015). While rhamnolipids are also able to denature proteins, they are considered weak binders, and typically have a reduced denaturing ability when compared to many other surfactants (Otzen, 2017). This may explain why biofilms exposed to SDS had lower

diversity than those in the rhamnolipid treatments or the controls. SDS also had the lowest amount of OTUs within the top 80% of taxa present in the treatments. We believe that the low diversity, and community domination by few taxa, demonstrates that SDS exposure creates an environment inhospitable to many potential specialist taxa. The few remaining and insensitive taxa can then thrive as more resources are available. Following our findings, these taxa would include *Brevundimonas terrae,* and an *Acinetobacter* and *Flavobacterium* spp. While the rhamnolipid treated flumes also had less diversity than the controls, the rhamnolipid treatments still appeared to favor more taxa and a higher diversity than the SDS treated flumes.

Extracellular Enzyme Activity

Our experiment showed that substantial changes in biofilm EEA can occur after surfactant exposure. Although phosphatase activity, or the separation of phosphate groups from a larger compound such as an acid or protein (Margalef *et al.,* 2017), generally decreased after surfactant exposure, we did have similar levels of activity between the control and SDS 150 flumes. Examination of the bacterial communities within SDS 150ppm flumes and control flumes show several parallel genera present that are not as common in the other systems, including *Nocardioides.* Treatments were laid out randomly across our experimental flumes within the experimental setup, and water was allocated to all systems from the same source, which minimized potential experimental alterations by relative location. We thus assume that all changes in community composition and EEA refer to the actual biofilm response to treatments. The presence of a low concentration SDS allowed for the development of bacterial genera like *Nocardioides* which typically have both acid and alkaline phosphatase (Xie *et al.,* 2017). However, the addition of high concentration SDS, or rhamnolipid at either a high or low concentration, reduced phosphatase activity.

Beta-glucosidase (rate-limiting enzyme that converts cellulase to glucose (Singh *et al.*, 2016)) and leucine aminopeptidase (enzyme that aids in the hydrolysis of leucine and other N-terminal residues at the end of peptides and proteins (Matsui *et al.*, 2006)) activities were also affected by surfactant exposure. Previous research has shown that rhamnolipids can act as an enhancer for the enzyme beta-glucosidase (Zhang *et al.*, 2009; Yao *et al.*, 2020). Rhamnolipids are able to protect proteins and limit the degradation of cellulase (Zhang *et al.*, 2009; Wang *et al.*, 2011). Yin *et al*. (2019) showed that the presence of rhamnolipid can increase genes related to leucine and amino acid synthesis. Addition of rhamnolipid to our flumes likely protected cellulase and leucine in the system which led to enhanced beta-glucosidase and leucine aminopeptidase activity. Beta-glucosidase is also known for its ability to degrade *Pseudomonas aeruginosa*, a rhamnolipid producing bacteria (Banar *et al.*, 2019; Pirlar *et al.*, 2020). The flumes treated with rhamnolipid had a higher abundance of *P. aeruginosa* when compared with the other flumes. Therefore, we assume that the higher beta-glucosidase levels within the rhamnolipid flumes were likely caused by a combination of the presence of rhamnolipid to act as an enhancer, and more abundant *P. aeruginosa.* However, we observed somewhat contrasting results in the SDS treated flumes, where a decrease in enzyme activity occurred. SDS is an anionic surfactant that can be used in denaturation of polypeptides (Bhuyan, 2009; Schlager *et al.*, 2012). SDS is commonly used in an analysis called SDS-polyacrylamide gel electrophoresis (SDS-PAGE) where an SDS infused gel is used to separate proteins and enzymes based on their molecular weight. This analytical method has been used with experiments examining beta-glucosidase previously (Laemmli, 1970; Bai *et al.*, 2013), which demonstrates that SDS is able to denature beta-glucosidase. It is possible that the denaturing ability of SDS has degraded beta-glucosidase

and leucine aminopeptidase, leading to reduced enzyme activity, and likely also reduced biofilm metabolic activity.

Conclusion

Our experiment successfully showed that both the biologic surfactant rhamnolipid and the synthetic surfactant, SDS, can significantly affect a mixed freshwater microbial biofilm in terms of community composition and metabolic activity. Rhamnolipids acted as an enhancer for beta-glucosidase and leucine aminopeptidase, increasing metabolic activity within the biofilm, while SDS likely denatured the enzymes and reduced metabolic activity within the biofilm. Both surfactants decreased the diversity of the biofilm bacterial community. There were unique genera in the biofilms of each treatment, including the controls which demonstrates that the presence of either surfactant creates an environment that is inhospitable to sensitive taxa. These results highlight two important areas of concern. First, rhamnolipids may not be a suitable alternative to SDS for use in industrial and medical biofilm control because of its lower effectiveness. Second, in natural ecosystems where biofilms are necessary for key ecosystem processes, such as degradation of organic matter, the presence of these surfactants, (for example through the release from WWTPs) may lead to negative effects on microbial biofilms. Altered biofilms may have decreased nutrient uptake efficiency due to shifts in their metabolic activity. This could have negative implications for the aquatic ecosystem as a whole, such as reduced denitrification.

Experimental Procedures

Experimental Set up

We set up 14 recirculating experimental flumes near Lunz Am See, Austria (following Roche *et al.*, 2017). Each flume was 2.5m long and had an approximate slope of 0.8%. In each

flume, ~5L of water recirculated, which was renewed with unfiltered lake water twice per week. We added rhamnolipid (R90-100G, Sigma-Aldrich, Darmstadt, Germany) and SDS (sodium dodecyl sulphate, Sigma-Aldrich, Darmstadt, Germany) as treatments of 150 and 300 ppm to three flumes each (hereafter referred to as either rhamno 150, rhamno 300, SDS 150, or SDS 300), while three flumes acted as controls. After the first week, 5 ug L^{-1} of Phosphorus in the form of Na_2HPO_4 was also added to help growth. Surfactant concentrations were chosen to represent the effective dose of rhamnolipid, as well as the double of that. Due to malfunction of one flume, *n* for the rhamnolipid treatment was reduced to two. Treatments were randomly placed among flumes, and light averaged 9.5 umol m^{-2} s^{-1}, with a light cycle of 12:12 and 18:6 hours of light versus dark early and later in the experiment., Each flume was equipped with microscope slides (6) and clay tiles (8) to quantify biofilm growth.

We measured pH, DO (mg/L), water temperature (° C), and flow (by bucket measurement) every other day to ensure stable conditions. Twice per week we analysed dissolved organic carbon (DOC), ammonium (NH_4), NO_x, and PO_4 of flume water to ensure they remained stable. In addition, water temperature was monitored by automatic loggers in drainage tanks. After 32 days the experiment was stopped and biofilm samples retrieved from the flumes for further analysis.

Sample Collection

Two microscope slides were removed from each flume and placed in 120mL Schott bottles with oxygen sensors. Bottles were filled with filtered lake water and sealed to remove all air bubbles. Three control bottles with no slides were also set up to test for potential activity within the filtered lake water. All bottles were placed in an Imago 500 Controller Environmental

Chamber (Snijders Scientific B.V., Tilburg, Netherlands) using a 12:12 light cycle for 72 hours. Oxygen measurements were taken at the start and end of each light and dark cycle to obtain data on GPP and CR.

We determined AFDM by weighing 2 clay tiles per flume after drying (9h at 70 ^{o}C), and combustion (4h at 450 ^{o}C). AFDM was obtained by subtracting the remaining ash weight from the dry weight. Two tiles from SDS 150 flumes were damaged during drying, and AFDM could not be determined. Biofilms covering two tiles per flume were then scraped into sterilized and pre-weighed 2mL Eppendorf tubes using a sterile razor blade, lyophilised (-0.001mbar; -76 □) and then analysed on a Thermo Scientific FLASH 2000 Elemental Analyser (Thermo Fisher Scientific GmbH, Bremen, Germany) to quantify carbon (% C) and nitrogen (% N) content. Quantities of C and N by mass were then examined in ratios to identify changes in ecological stoichiometry (the relationship between elements, energy, and organisms and their interactions in an ecological system (Sterner and Elser, 2002; Van de Waal *et al.*, 2018)).

EEA of β-D-1,4-glucosidase (glucosidase), phosphatase, and leucine-aminopeptidase were measured in the biofilms following the method described in Romaní and Sabater (2001). Four mL of sterile water was added to approximately 0.5 g of biofilm per flume that was scraped from the tiles. The mixture was homogenized by shaking. EEA was measured via artificial substrates: Glucosidase and phosphatase using 4-methylumbelliferyl (MUF)- substrates (MUF-β-D-glucopyranoside and MUF-phosphate, respectively (Sigma-Aldrich, Darmstadt, Germany)) and leucine-aminopeptidase using a 7-amino-4-methylcoumarin (AMC)- substrate (L-leucine-AMC, Sigma-Aldrich, Darmstadt, Germany). Artificial substrates were added to the homogenate at a final concentration of MUF-glucosidase; 135 mg L^{-1}, MUF-phosphatase; 102.46 mg L^{-1}, and AMC-leucine-aminopeptidase; 129.94 mg L^{-1} and incubated for 1 hour in the dark. Fluorescence

was measured at the beginning and end of the incubation with a platereader (Varioskan Flash, Thermo Fischer Scientific, Vaanta, Finland) at 365/455 nm excitation/emission wavelengths for the MUF-substrates and 380/440 excitation/emission wavelengths for the AMC-substrate.

Two microscope slides per flume were scraped using sterile procedures into a sterile 1.5mL microcentrifuge tube for 16s rRNA analysis. DNA was immediately extracted from collected samples using the Dneasy Power Soil Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's methodology. We included internal replicates per flume (n=2), and experimental controls were used to control for any potential unaccounted sample contamination. Extracted products were sent to LGC Biosearch Technologies (LGC Genomics GmbH, Berlin, Germany). DNA was quantified using gel electrophoresis before amplifying the V3-V4 region of the 16s rRNA gene with polymerase chain reactions using 341F and 785R primers. Resultant DNA were sequenced using the Illumina Mi-Seq platform (300 bp paired-end reads) for amplicon creation (sequencing results will be made available online after manuscript acceptance and publication).

We used Mothur version 1.44.3 (Schloss *et al.*, 2009), and the Mothur MiSeq SOP (Kozich *et al.*, 2013) with some modification, to analyze returned reads. A maximum homopolymer of 8 was chosen due to the limitation of the reference database (SILVA version 132) with a 97% similarity and above required for identification of operational taxonomic units (OTUs). UCHIME (Edgar *et al.*, 2011) was also used to identify and remove chimeric fragments. Sequences that could not be directly identified through SILVA, were manually run through NCBI's BLAST (Madden, 2002). We were able to identify all but one OTU with 97% similarity; OTU005 is thus reported as 'unnamed' in this study.

Data analysis

All data, except the 16s rRNA and C:N results, met assumptions of normality and homogeneity of variance. We considered $p < 0.05$ as significant and analyzed data using R version 4.0.3 (R Core Team, 2020) and the R packages car (Fox and Weisberg, 2019), vegan (Oksanen *et al.*, 2020), ggplot2 (Wickham, 2016), and entropart (Marcon and Herault, 2015). Non metric multidimensional scaling analyses (NMDS) were run using the PAST 3 software (Hammer *et al.*, 2001). Due to replication error early in the experiment only the controls and 300 ppm concentration variables were used in statistical difference testing. All GPP and CR data were normalized to mg O_2 in 120mL per mg of biofilm before being analyzed. C:N data was normalized to mass (mg C/N per mg biofilm). A one way analysis of variance (ANOVA) analysis was used to test for significant differences among treatments and the controls for the GPP, CR, and AFDM. A Kruskal-Wallis test was used to test for significant differences among the C:N ratios. Coefficients of variation were also calculated for individual flumes for each analysis.

Changes in EEA were analysed through both a distance based redundancy analysis (DRA) and three one-way ANOVAs, one per extracellular enzyme. All the data was used within the DRA and only the maximum concentrations of each chemical and control were used for analysis in the ANOVA. Where appropriate, we applied a Tukey HSD post-hoc test to the ANOVA output.

Bacterial OTUs were analyzed for alpha and beta diversity as well as differences in the overall bacterial community composition. We used the Chao1 (Chao, 1984) and the Shannon-Wiener Diversity Indices to quantify bacterial Alpha diversity (Jost, 2007). Chao 1 and Shannon-

Wiener results were then analyzed using an ANOVA followed by a Tukey HSD post-hoc test to identify differences in diversity among treatments. Following this, all singletons and doubletons were removed and the data was rarefied based on the smallest number of sequences identified within a sample. Beta diversity was then analysed using a non-metric multidimensional scaling analysis (NMDS) on all data present to identify differences in the biofilm bacterial community among treatments and controls. An analysis of similarities (ANOSIM) was performed with 999 permutations to identify if these differences were significant. Families and genera were compared graphically, focusing on the top 80% of OTUs identified within the biofilms.

significant differences).

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Conflict of Interest

The authors declare no conflict of interest.

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Table and Figure Legends

Table 1 Coefficients of variation for AFDM, GPP, and CR data. Data collected from samples of biofilm grown in either the surfactant SDS (sodium dodecyl sulphate) or rhamnolipid for 32 days.

Table 2 Biofilm OTUs per surfactant treatment and their dominant genera. Biofilms were grown in either SDS (sodium dodecyl sulphate) or rhamnolipid for 32 days.

Figure 1 GPP (A), CR (B) and AFDM (C) of microbial biofilms exposed to SDS (sodium dodecyl sulphate) and rhamnolipid for 32 days at 150 and 300ppm. Controls are shown in brown, rhamno 150 in dark green, rhamno 300 in light green, SDS 150 in dark blue, and SDS 300 in light blue.

Figure 2 Carbon (A), Nitrogen (B) and C:N ratios (C) of microbial biofilms exposed to SDS (sodium dodecyl sulphate) and rhamnolipid for 32 days at 150 and 300ppm. Controls are shown in brown, rhamno 150 in dark green, rhamno 300 in light green, SDS 150 in dark blue, and SDS 300 in light blue.

Figure 3 Biofilm bacterial diversity from 16s rRNA sequencing of biofilm exposed to SDS (sodium dodecyl sulphate) or rhamnolipid after 32 days at 150 and 300ppm. A) shows Shannon Diversity, B) shows an NMDS depicting the community differences in treatments when examining all OTUs present within each sample after rarefication. Controls are shown in brown, rhamno 150 in dark green, rhamno 300 in light green, SDS 150 in dark blue, and SDS 300 in light blue.

Figure 4 EEA of phosphatase (A), glucosidase (B), and leucine aminopeptidase (C) of biofilms after exposure to either SDS (sodium dodecyl sulphate) or rhamnolipid over 32 days at 150 and 300ppm. Controls are shown in brown, rhamno 150 in dark green, rhamno 300 in light green, SDS 150 in dark blue, and SDS 300 in light blue (*indicates significant differences).

Table 1

Treatment	Ash free dry mass	Gross primary productivity	CR	C:N
SDS 150	0.70	0.67	.92	0.373
SDS 300	0.11	0.626	1.09	0.643
Rhamno 150	0.60	0.34	0.021	0.0875
Rhamno 300	0.32	0.52	0.59	0.0698
Control	0.59	0.61	1.56	0.569

Table 2

Surfactant	Conc. (ppm)	Total OTUs present	OTUs in top 80% per treatment	OTUs lost compared to control	OTUs gained compared to control	Dominant genera in top 80% of all taxa present						
Rhamnolipid	150	145	10	58	25	*Brevundimonas*	*Otu005*	*Pseudomonas*	*Arthrobacter*	*Flavobacterium B*	*Prosthecobacter*	*Nocardioides*
Rhamnolipid	300	159	14	44	36	*Brevundimonas*	*Otu005*	*Pseudomonas*	*Arthrobacter*	*Flavobacterium B*	*Prosthecobacter*	
SDS	150	176	7	27	37	*Brevundimonas terrae*	*Acinetobacter*	*Flavobacterium A*	*Paucibacter*	*Flavobacterium B*	*Prosthecobacter*	
SDS	300	130	6	73	23	*Brevundimonas terrae*	*Acinetobacter*	*Flavobacterium A*	*Prosthecobacter*	*Flavobacterium B*	*Prosthecobacter*	*Sphingomonas*
Control	0	203	15	--	--	*Exiguobacterium*	*Acinetobacter*	*Arenimonas*	*Stenotrophomonas*	*Nocardioides*		

Figure 1

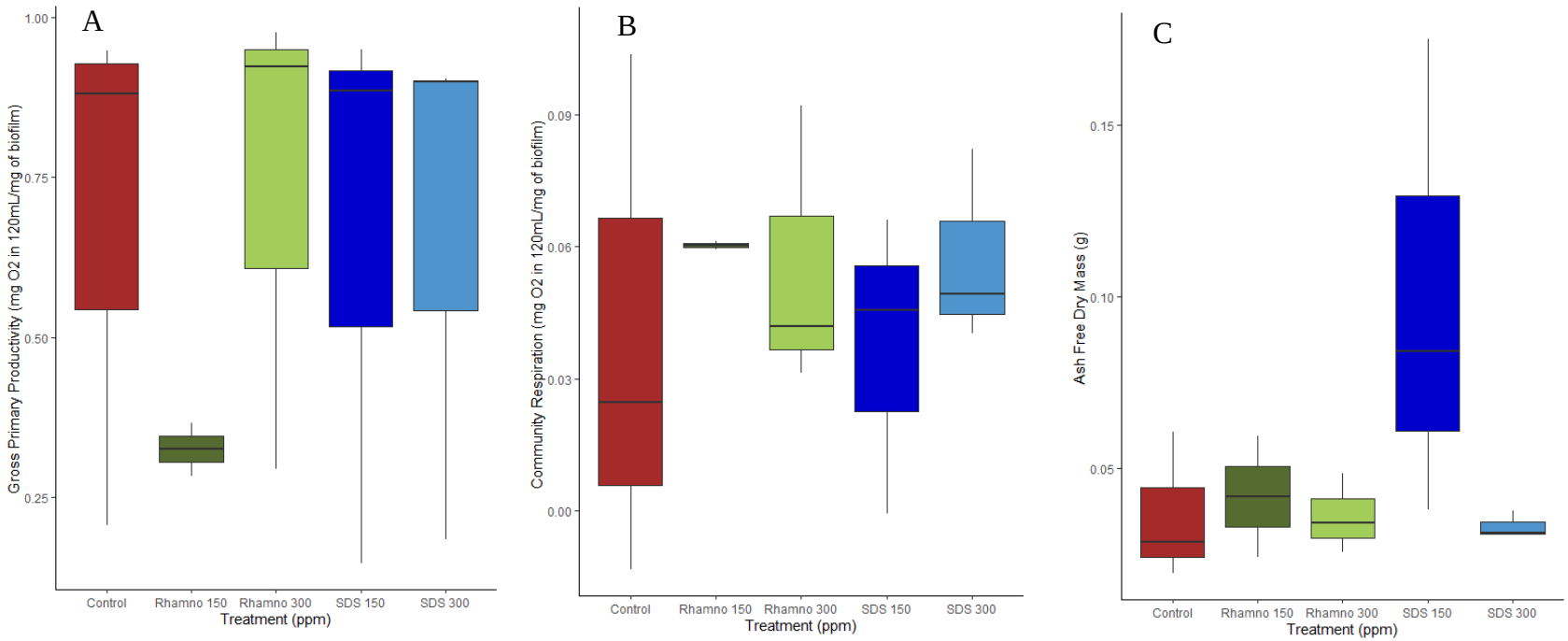

A
Gross Primary Productivity (mg O2 in 120mL/mg of biofilm)
1.00
0.75
0.50
0.25
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
B
Community Respiration (mg O2 in 120mL/mg of biofilm)
0.09
0.06
0.03
0.00
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
C
Ash Free Dry Mass (g)
0.15
0.10
0.05
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)

Figure 2

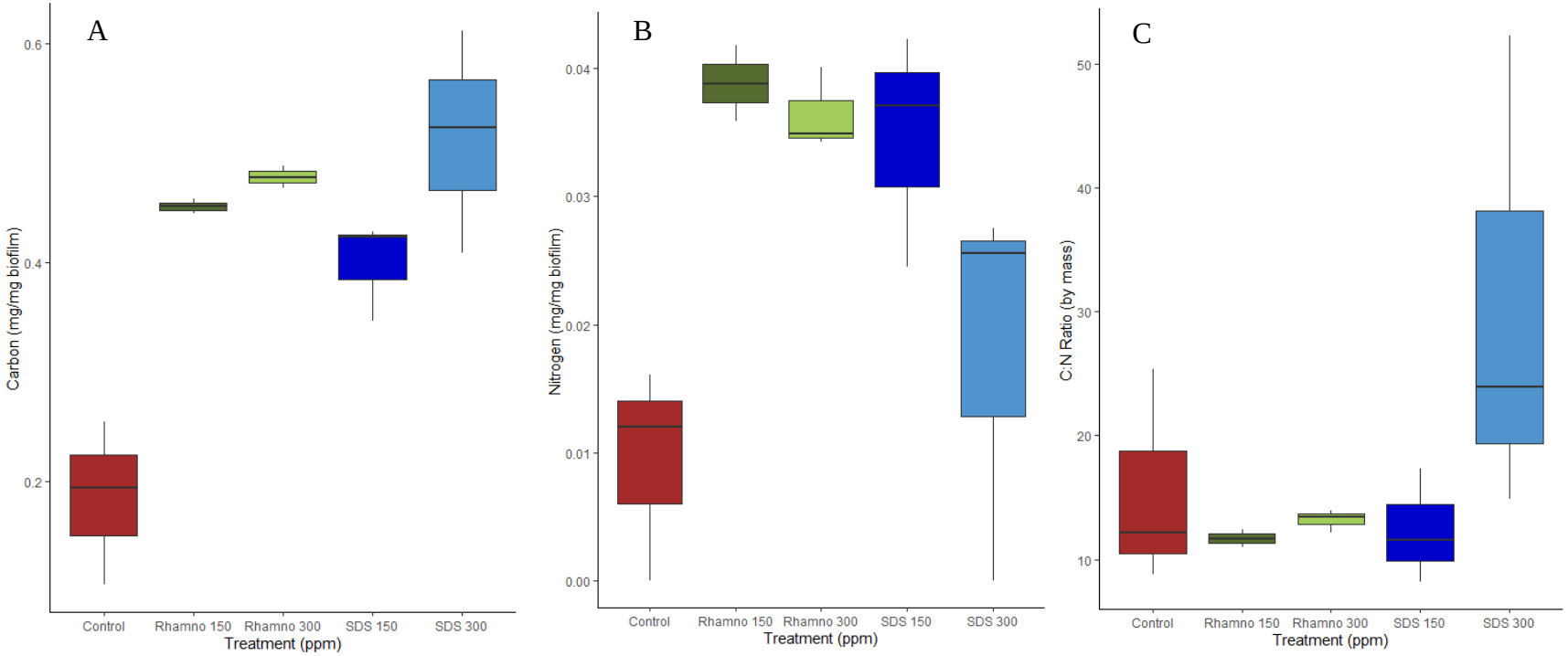

A
Carbon (mg/mg biofilm)
0.6
0.4
0.2
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
B
Nitrogen (mg/mg biofilm)
0.04
0.03
0.02
0.01
0.00
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
C
C:N Ratio (by mass)
50
40
30
20
10
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)

Figure 3

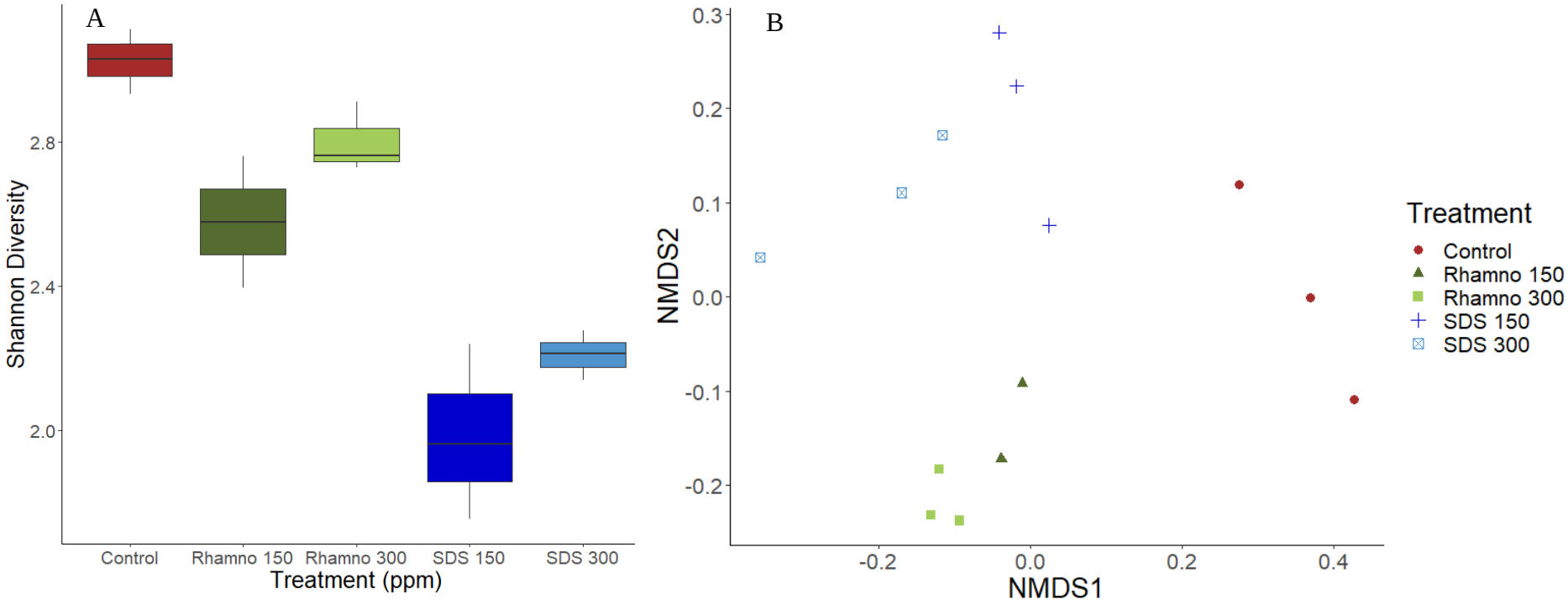
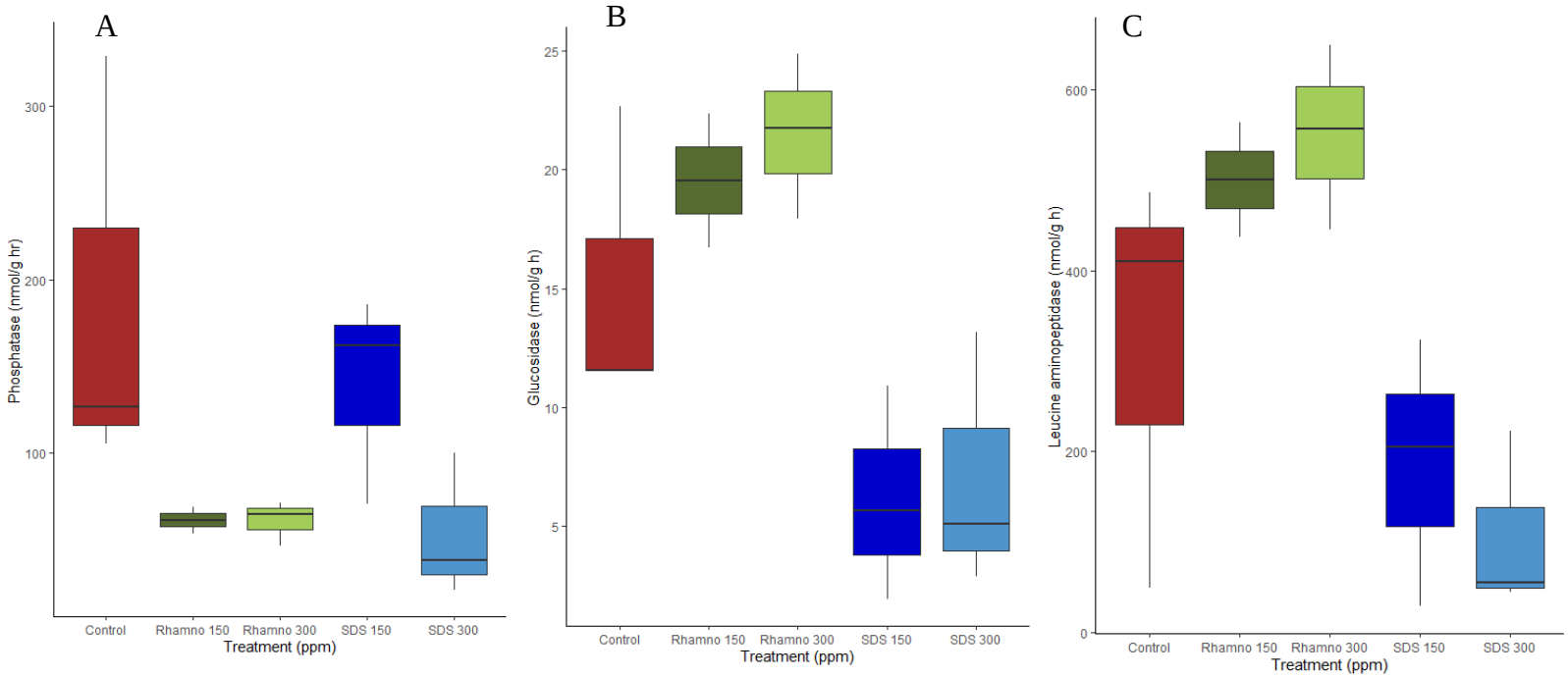

A
Shannon Diversity
2.8
2.4
2.0
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
B
NMDS2
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.2
0.0
0.2
0.4
NMDS1
Treatment
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300

Figure 4


A
Phosphatase (nmol/g hr)
300
200
100
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
B
Glucosidase (nmol/g h)
25
20
15
10
5
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)
C
Leucine aminopeptidase (nmol/g h)
600
400
200
0
Control
Rhamno 150
Rhamno 300
SDS 150
SDS 300
Treatment (ppm)