Long-term effects of dissolved nutrient and organic carbon loading on the efficiency of in-stream nutrient uptake and metabolic processes

Master thesis

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ABSTRACT

Low order streams are very important in terms of reducing nutrient downstream transport by biotic and abiotic nutrient retention. Through the extensive use of organic and inorganic fertilizers in agriculture, natural equilibrium of stream ecosystems between production and decomposition has changed and thus, dramatically affected the self-purification capacity of streams. For an improved management of agricultural streams it is necessary to investigate the effects of a long term enrichment of inorganic and organic fertilizers and how they affect water quality and ecosystem functions. This study was designed to understand how algal and bacterial biomass production and activities including nutrient uptake and decomposition in biofilms change over time with continuous nutrient enrichment for different substrate types in an experimental setting. We were also interested to find out at which concentration a saturation can be reached, wherefore we conducted our experiment with moderate and high nutrient levels. The experiment lasted over two months including four weeks of colonization phase, four weeks of enrichment phase and two weeks recovery phase. Results have shown that chl-a concentrations strongly increased with moderate and high nutrient enrichment but saturation was not reached. Also bacterial biomass, where differences were less distinctive, did not reach a saturation of biomass production and nutrient uptake. Differences were generally less affected by nutrient enrichment. Furthermore, investigations of the release of extracellular enzymes indicated a sign of saturation referring to the release of phosphatase and showed an adaption to enrichment through a higher decomposition of algal cells but also a stimulated decomposition of cellulose compounds.

Overall, this study revealed, that biofilms adapt nutrient uptake and metabolic processes in moderate and high enriched flumes probably due to altered structural and functional characteristics. Nevertheless, chosen nutrient levels could not fully cause a saturation.

Key words: agricultural streams, extracellular enzymes, decomposition, nutrient uptake, algal and bacterial biomass

ZUSAMMENFASSUNG

Kleinere Fließgewässer sind von besonderer Bedeutung in Bezug auf die Reduzierung des Nährstofftransports durch abiotische und biotische Nährstoffretention. Durch den intensiven Einsatz von organischen und anorganischen Düngemitteln in der Landwirtschaft hat sich das natürliche Gleichgewicht der Ökosysteme stark verändert und somit auch ihre Selbstreinigungskapazität. Für eine bessere Bewirtschaftung von landwirtschaftlich beeinflussten Fließgewässern müssen die Auswirkungen einer langfristigen Anreicherung anorganischer und organischer Düngemittel sowie deren Auswirkungen auf die Wasserqualität und die Funktionalität des Ökosystems untersucht werden. Diese Laborstudie, welche unter regulierten Licht- und Nährstoffverhältnissen stattgefunden hat, soll generell aufzeigen, wie sich eine permanente Nährstoffanreicherung über vier Wochen auf den Anstieg der Algen- und Bakterienmasse sowie deren Aktivität in unterschiedlichen Substrattypen auswirkt.

Um eine eventuelle Sättigung des Algen- und Bakterienwachstums als auch deren Nährstoffaufnahme festzustellen, wurden zwei unterschiedlich hohe Nährstoffkonzentrationen gewählt. Als Referenz für die Höhe der Konzentrationen orientierte man sich einerseits an einer moderaten Weidewirtschaft als auch an intensiv betriebenen Ackerbauflächen im Weinviertel. Die Ergebnisse zeigten, dass Chl-a sowohl in der moderaten als auch in der hohen Anreicherungsgruppe stark anstieg, eine Sättigung jedoch nicht erreicht wurde. Auch die bakterielle Biomasse, bei der die Unterschiede zwischen den Gruppen weniger ausgeprägt waren, erreichte keine Sättigung und war im Vergleich zur Algenbiomasse deutlich geringer von der Nährstoffzugabe beeinflusst. Der stetig wachsende Biofilm führte letztendlich dazu, dass die Nährstoffaufnahme in den Versuchsgruppen deutlich anstieg. Die Untersuchung zur Freisetzung extrazellulärer Enzyme zeigte zudem eine mögliche PO4-P Sättigung und einen erhöhten Abbau an komplexem organischen Material, welche durch die Zugabe von leicht verfügbarem Acetat möglicherweise begünstigt wurde.

Zusammenfassend lässt sich sagen, dass sich Biofilme wahrscheinlich aufgrund von veränderten strukturellen und funktionellen Eigenschaften an hohe Nährstoffkonzentrationen anpassen, was sich unter anderem anhand einer erhöhten Nährstoffaufnahme als auch an einem gesteigerten Stoffwechsel nachvollziehen lässt.

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1. Introduction

During the last century, anthropogenic pressures like point and non-point pollution have increased concentrations of nitrogen, phosphorous and dissolved organic carbon in streams with crucial impacts on water quality and ecosystem functions (Kunikova, 2013; Vitousek et al., 1997). Algal and bacterial biomass production and activities (including primary production, nutrient uptake and decomposition) are affected differently by nutrient and organic carbon enrichment, leading to an imbalance between organic matter production and decomposition and a decrease in the self-purification capacity of the aquatic system (Sabater et al., 2011).

While point source pollution could be reduced in Austria during the last few years, agricultural land use still poses a growing issue in water pollution (Mateo-Sagasta et al., 2017). Un-impacted low order streams are characterized by low concentrations of inorganic nutrients and dissolved organic carbon and, thus, low primary and secondary production, which are usually at equilibrium (Waters et al., 2005). This natural equilibrium of ecosystems - which implies the equilibrium between production and decomposition - has changed through the extensive use of organic and inorganic fertilizers in agricultural catchments. With the excess supply of inorganic nutrients and optimal light conditions, a result of the frequent removal of riparian forests in agricultural catchments, primary production has increased dramatically in agricultural streams. Examples of eutrophicated streams can be found in agricultural catchments all over the world (Mateo-sagasta, 2010; Withers et al., 2014). The increased supply of nutrients and organic matter has also crucial impacts on microbial abundances and activities. Experiments have shown a strong positive reaction of both algal and bacterial growth as well as of the release of extracellular enzymes necessary for organic matter degradation ongoing with high inorganic nutrient availability (Sabater et al., 2011). Moreover, excess inorganic nutrient loading may saturate biotic nutrient demand causing higher downstream nutrient transport which increases the risk of transmitting eutrophication downstream (Vitousek et al., 1997).

In addition to inorganic nutrient loading, agricultural streams are furthermore affected by increased amounts of dissolved organic carbon due to the use of organic fertilizers and agricultural practices (Williams et al., 2010). Dissolved organic carbon has a very important role in aquatic ecosystems as it represents the main energy source for heterotrophic bacteria (Stanley et al., 2012). Quantity and quality of organic carbon sources are therefore crucial factors influencing microbial activity.

While un-impacted headwater streams are mainly characterized by terrestrially derived organic carbon, organic fertilizers provide streams with highly bioavailable carbon compounds (Graeber et al., 2012). Such fractions are preferred by heterotrophic bacteria as they can easily be metabolized (Lutz et al., 2012), implicating a further increase in microbial activities. The self-purification of streams is a process, which allows aquatic systems to take up a certain amount of dissolved inorganic nutrients and organic matter through abiotic and biotic retention. One of the most important processes, especially in low-order streams, is the biotic nutrient retention. Biofilms, which are a conglomerate of algae, bacteria and fungi in a poly-saccharide matrix (Flemming and Wingender, 2010; Sutherland, 2001) colonizing all surface areas in the stream in both, the channel and the hyporheic zone, are hot spots for nutrient uptake and retention and mitigate downstream transport of nutrients (Besemer et al., 2013; Bukaveckas, 2007; Romaní et al., 2004). In stream ecosystems, the extent of nutrient retention therefore depends on the abundance, composition and activity of the benthic community within the stream channel which may strongly vary between epilithic and epipsammic biofilm (Payn et al., 2005; Romaní and Sabater, 2001, Lock 1993). Due to adaptions of benthic communities to pollution in both biomass production and enzymatic activities, streams show a certain resilience to pressures of inorganic nutrient and organic carbon loading. However, if pressures are too high, the above mentioned ecosystem functions will be reduced with crucial effects on both the integrity and the ecological state of the stream ecosystem.

Former studies have often focused on the influences of short-term nutrient enrichment or long term enrichments of single nutrients but less was known about the influences of combined organic and inorganic chronic pressures (Bernot and Dodds, 2005; Sabater et al., 2011). For a better management of agricultural streams, the question arises how long-term enrichments of inorganic nutrients and dissolved organic carbon affect benthic communities and processes in stream ecosystems and, furthermore, how long it takes them for recovery after the cessation of the enrichment.

1.1 Thematic background

1.1.1 The effects of nutrient and organic matter enrichment on benthic algal and bacterial biomass

In headwater streams, the majority of stream processes takes place at the water-sediment interface. In general, enhanced nutrient concentrations support the growth of both benthic algae and bacteria

(Sabater et al., 2011). Often, there are no clear correlations between benthic chlorophyll-a and nutrient background concentrations in field studies (or with nutrient diffusing substrata) due to other influencing factors such as light or hydrology (e.g. Kohler et al., 2012). However, significant increases of periphyton biomass in nutrient enriched stream sections compared to reference sections in specific under optimal light conditions were determined (e.g. Hill et al. 2011; Sabater et al., 2001). Other studies found diverse results of nutrients on both periphyton and bacterial biomass depending on the respective substrate. They observed a clear increase of chlorophyll-a and bacterial density in epilithic biofilms due to nutrient enrichment, whereas there was no increase of chlorophyll-a and bacterial density in epipsammic biofilms (Anna M. Romaní et al., 2004; Sabater et al., 2011).

In addition to nutrients, benthic bacteria are also affected by the amount and the quality of organic matter, in specific of dissolved organic carbon. Organic carbon normally occurs in streams as allochthonous carbon derived from the watershed or as autochthonous carbon derived from algal exudates (Jonsson et al., 2001) These two types can be distinguished due to their source, but also due to their molecular weight. Whereas terrestrial derived organic material mainly consists of refractory compounds (humic and fulvic acids), autochthonous organic material (e.g algal-based-detritus) is rather characterized by low molecular weight which can be assimilated without great energy input (McKnight et al., 2001).

Dissolved organic carbon is a part of organic material and represents the major part of the organic carbon pool. In pristine ecosystems, terrestrial and aquatic primary production is the main factor contributing new labile DOC to aquatic systems (Stanley et al., 2012). This has changed with the intensive use of organic fertilizers, but also through the influx of sewage plants which lead to an additional input. Labile DOC - which is often observed to be the limiting factor for bacterial metabolism - is of great biological importance as it can immediately be assimilated by heterotrophic bacteria and thus has crucial effects on their growth and respiration (Massicotte and Frenette, 2013; Stanley et al., 2012). It is characterized by a high bacterial growth efficiency, which defines the part of DOC which is assimilated to bacterial biomass (Eichinger et al., 2010).

In addition to the above-mentioned effects of nutrient and organic matter enrichment on algae and bacteria, there are several studies, which have investigated positive but also negative interactions between benthic algae and bacteria in matters of nutrient competition and carbon cycling. A positive interaction, for example, is the release of dissolved organic carbon by photosynthesizing

algae which can be utilized by bacteria, while bacteria supply algae with nutrients from organic matter mineralization (Bell, 1983; Larsson and Hagström, 1979). Rier and Stevenson (Rier and Stevenson, 2001) observed, that these positive interactions between bacteria and algae mainly appear under oligotrophic situations, while the linkage between algae and bacteria is less strong under eutrophic conditions. However, not only DOC poses an important interaction between bacteria and algae, but also their competition for inorganic nutrients. Numerous studies have already proven, that heterotrophic bacteria show a high affinity to phosphorous and thus are able to compete with algae (Currie and Kalff, 1984).Their fast growth rates and high surface area to volume ratio are characteristics, which provide them a clear advantage in contrary to algae, which are rather characterized by their slow growth rate. This competition was mainly observed in P-limited systems (Rhee, 1972).

1.1.2 The effects of dissolved nutrients and organic carbon on the production of extracellular enzymes by algae and bacteria

Biofilms play an important role in the self-purification of streams by removing inorganic and organic nutrients from water bodies through biotic nutrient uptake (Liu et al., 2014; Sabater et al., 2002). Especially headwater streams – which are dominated by benthic biofilms - are of particular importance as they have a high surface area-to-volume ratio resulting in an enhanced biochemical processing and nutrient uptake (Alexander, Boyer, Smith, Schwarz, and Moore, 2007). Nevertheless, headwater streams are often nutrient limited as they are dominated by high molecular compounds (e.g allochthonous material) which first need to be degraded into smaller units before they can be taken up by heterotrophic bacteria (Francoeur and Wetzel, 2003; Graça and Canhoto, 2006).

Extracellular enzymes, like phosphatase, peptidase, glucosidase, xylosidase and cellobiohydrolase, play an important role in the microbial decomposition of organic matter as they function as catalysts for the breakdown of organic molecules containing carbon, nitrogen and phosphorus bonds. As enzyme production is an energy-intensive process, the production and release of extracellular enzymes by bacteria is mainly determined by the amount of bioavailable nutrients in the water column. However, the effects of increased nutrient availability on the release of extracellular enzymes differ among the various enzymes and furthermore is strongly affected by the functional and structural characteristics of the various biofilms (Romaní et al., 2004). Enzymes, which are responsible for splitting P from organic molecules, such as phosphatase are primarily

released when dissolved nutrients are scarce. This could be demonstrated in several experiments showing a negative correlation between a certain nutrient and its corresponding enzyme Koch, 1985;)(Koch, 1985; Sinsabaugh and Moorhead, 1994). However, enzymes which convert complex sugars into simpler ones, like glucosidase, cellobioase, peptidase or xylosidase, have been observed to increase at enhanced nutrient availability due to the stimulated microbial decomposition (Sabater et al., 2011).

Due to their use of organic substrates as energy source, heterotrophs are the main producers of extracellular enzymes (Allison and Vitousek, 2005; Battin et al., 2009; Rier et al., 2014; Romaní et al., 2004; R. L. Sinsabaugh et al., 1994). But algae also need to be considered regarding the production of phosphatase. Algae have been observed to be the main producers of phosphatase as their growth is often constrained by the availability of orthophosphate (Berman, 1970; Boavida and Heath, 1984). Based on this knowledge, a high production of a certain nutrient-generating enzyme may indicate a limitation of this nutrient (H. Liu et al., 2012; Sabater et al., 2011; Wagner et al., 2014).

In a long-term study, Sabater et al. (2011) investigated the effects of moderate nutrient loading on the extracellular enzyme production in a forested stream. They observed that biofilm phosphatase decreased in the enriched stream section, which might be interpreted as a saturation of algae normally representing the main phosphatase producers. In contrast, peptidase, glucosidase, xylosidase and cellohydrobiolase showed an increase in the enriched reach. Whereas the higher activity of peptidase and beta-glucosidase could result from enhanced primary production and thus the greater amount of low-molecular compounds, xylosidase and cellohydrobiolase showed an increase due to stimulated decomposition of cellulose and lignin compounds (Gulis et al., 2004; Sabater et al., 2011). A further important observation was that the bacterial production of extracellular enzymes differed among substrate type. The epipsammon, which is generally characterized by low algal biomass production due to high sediment turnover, most likely showed higher peroxidase and cellobiohydrolase activities, which are closely related to the decomposition of terrestrial organic matter. In contrary to the epipsammon, the epilithon is mainly characterized by higher algal biomass production, which can furthermore be supported by enzymes leated to the break-down of algal biomass e.g. peptidase and ß-glucosidase (Allison and Vitousek, 2005; Gulis et al., 2004; Anna M. Romaní et al., 2004; Sabater et al., 2011).

Due to its strong influences on microbial activities and thus on the self-purification capacity of streams, several studies have focused on labile organic carbon loading. Lutz et al. (2012) investigated short-term effects of labile DOC on microbial decomposition with the observation that decomposition rates decreased with increasing DOC addition. This could imply that labile dissolved organic carbon may saturate bacterial carbon demand and thus reduce further decomposition of refractory compounds. In contrast, other studies have demonstrated the linkage between higher DOC concentrations to higher DOC uptake and leaf-litter decomposition which could be explained by the demand of increased bacterial densities (Wilcox et al., 2005; (Massicotte and Frenette, 2013; Tulonen, 2004). This positive effect could already be observed in several studies and is called "priming effect", which implies the utilization of labile algal-derived in order to gain energy for the degradation of more refractory compounds (Bengtsson et al., 2014; Kuzyakov, Friedel, and Stahr, 2000; Danger et al., 2013).

However, prevailing problems relating to the use of agricultural fertilizers should more support the idea of investigating effects of inorganic nutrient as well as organic carbon loading. As already mentioned in the chapter before, organic fertilizers provide streams with highly bioavailable forms, which do not require the release of extracellular enzymes. Experiments have shown, that labile DOC provided by algae could stimulate decomposition and increase DOC uptake, but with a continuously enrichment of highly bioavailable DOC, heterotrophs could also be saturated and thus inhibit further decomposition (Suberkropp et al., 2010).

1.1.3 Effects of nutrient enrichment on nutrient uptake

Nutrient uptake is one of the most important ecosystem functions providing information about the integrity of a system (Dodds, 2003; Young et al., 2008). Nutrient uptake is the capacity of streams to retain imported nutrients within the stream ecosystem. It can be divided into two categories, biotic and abiotic nutrient uptake. Abiotic uptake is characterized by physical-chemical processes like precipitation but also through sorption onto sediments, which represents a very important nutrient sink especially for phosphorous (Lottig and Stanley, 2007). The effectiveness of how nutrients are adsorbed on particles is mainly determined by the sediment grain size, with smaller grains showing higher capacities to adsorb phosphorus (Lottig and Stanley, 2007).

Biotic nutrient uptake – which is characterized by uptake through algae, macrophytes, and bacteria - has the most important role in nutrient retention and consequently on water quality. Especially low order streams, where the available surface for nutrient uptake is high compared to the water column, are of particular importance for nutrient uptake. Benthic algae utilize inorganic nutrients like ammonia, nitrate and phosphate for their biomass production. Although heterotrophic bacteria are mainly known for their important function as decomposers and, thus, can provide parts of their nutrient supply themselves, they also have the ability to take up inorganic nutrients from the water column (Rier and Stevenson, 2002). This was mainly observed in situations, where organic substrates were limited (Rhee, 1972). But also under non-limiting conditions, certain bacteria may prefer inorganic nutrients (Danovaro, 1998).

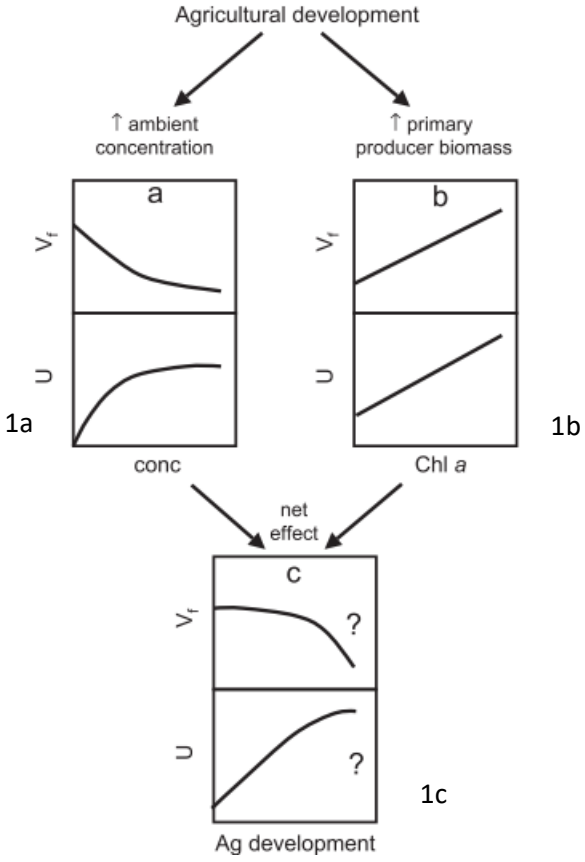

Figure 1: This figure shows a conceptual model by Niyogi et al .(Niyogi et al. 2004) dealing with the effects of agricultural development on nutrient uptake influenced by altered nutrient concentrations and primary producers biomass. (V_f = uptake velocity; U = uptake rate)

Biotic nutrient uptake is directly dependent on the nutrient demand of primary producers and heterotrophic microorganism, but also on prevailing nutrient concentrations (Bernot et al., 2006; Epa, 2006; Payn et al., 2005). In general, nutrient uptake is expected to follow a Michaelis-Menten saturation curve (C. Davis and Minshall, 1999). Studies have shown, that increasing nutrient concentrations lead to nutrient saturation and, consequently, to a reduction of in-stream nutrient uptake and retention (Bernot et al., 2006; Dodds et al., 2002). Thus, if nutrient concentrations exceed a certain threshold, nutrient uptake rates will reach a plateau (maximum uptake rates), while uptake velocities, which reflect the nutrient demand, will exponentially decrease to a minimum (Earl et al., 2006). Niyogi et al. (2004) expanded this uptake model by including the effects of increased algal growth through enhanced nutrient supply in agricultural streams (*Figure 1*).

The first graph (1a) follows the assumption, that with increasing nutrient concentration but constant biomass, uptake velocity will decrease and finally reach a saturation point (Horner et al., 1990).

On the other hand, graph 1b demonstrates a situation with increasing biomass under non-limiting nutrient concentrations, resulting in a permanent nutrient demand and uptake velocity. Modell 1c considers both, an increase in algal biomass but also in ambient nutrient concentration. As already shown in numerous studies, nutrient enrichment increases algal biomass and subsequently also leads to higher nutrient demand (Sabater et al., 2011). Nevertheless, if nutrient concentrations are too high, nutrient uptake will decrease and sooner or later reach a saturation point. This raises the question - in relation to model 1c - how uptake velocity and uptake rate will change and when saturation is finally reached.

2. Rationale

Stressors like pollution with organic and inorganic nutrients are still prevailing problems affecting our streams. In the past, realized restoration measures and management efforts to obtain higher water quality mainly concentrated on problems with inorganic nutrient loading, whereas organic carbon loading has not been considered as much (Stanley et al., 2012). Recent research projects focus more on the effects of inorganic and organic nutrient loading, but mostly in oligotrophic water bodies where nutrients are limited. Continuously polluted water bodies e.g. through the effluent of wastewater treatment plants or agricultural land use in highly productive areas are still poorly understood. This missing knowledge strengthened the idea of conducting an experiment, which solely focuses on the effects of combined chronic inorganic nutrient and organic carbon loading on stream community and benthic processes in continuously enriched streams. As already mentioned in the chapter above, factors like hydrology, light, substrate but also macrozoobenthos would have had strong influences on this investigation, which supported the idea of an experimental framework, where all influencing factors are regulated.

Based on the results of this study, it should be possible to obtain a better understanding of the structure and function of permanently enriched systems. Further protection measures which are based on these data could then help to re-establish the self-purification capacity of impacted streams.

2.1 Research questions

1) How does a continuous enrichment of dissolved inorganic nutrients and organic carbon over several weeks alter in-stream processes at the water sediment interface?

    a) How are the abundances of benthic algae and bacteria affected by the enrichment within different stream substrates? Do algal and bacterial abundances show a linear increase with increased enrichment or are there already saturation effects within the chosen nutrient levels? Do benthic algae and bacteria differ in their reaction to the nutrient enrichment?
    b) How is the production of extracellular enzymes affected by the enrichment and are there differences between epilithic and epipsammic biofilms? Do extracellular enzymes which are necessary for organic matter degradation (glucosidase, peptidase, xylosidase) differ from phosphatase in the reaction to increased nutrient concentrations?
    c) How is the in-stream uptake of ammonium and phosphate influenced? Is there a saturation of nutrient uptake with increasing nutrient enrichment?

2) How long does it take for the different processes to recover from the enrichment?

2.2 Hypothesis

- Algal and bacterial biomass in epipsammic and epilithic biofilm will increase with moderate and high long term enrichment of acetate, phosphate and nitrate
- Phosphatase in epilithic and epipsammic biofilm will decrease with moderate and high long-term enrichment of phosphate
- The release of peptidase, glucosidase and xylosidase will change with moderate and high long term enrichment of acetate, phosphate and nitrate
    - Peptidase, glucosidase and xylosidase will increase with moderate and high long term enrichment of acetate, phosphate and nitrate
    - Peptidase, glucosidase and xylosidase will decrease with moderate and high long term enrichment of acetate, phosphate and nitrate
- Microbial decomposition will increase with the addition of dissolved organic carbon
- Nutrient uptake will increase with increasing nutrient addition

3. Materials and methods

3.1 Study site

This study was carried out in experimental flumes (á 3 m long x 5 cm wide x 5 cm high) which were placed inside a hut nearby the WasserCluster Lunz in Lunz am See.

3.2 Flume setup

Drip rails were placed on 1,5x2 m wooden boards which were additionally fixed on a 3 m bench. To achieve a constant slope and a constant flow rate for all flumes, timber wedges with a height of 20 cm were placed underneath the construction. To avoid substrate aggregation, synthetical sponges á 10x10 cm were fixed directly at the inlet.

Each flume was filled up to a height of 3 cm with pre-cleaned fine gravel of 2 mm grain size and laid out with 36 Epilithic á 19,77 cm^2, which served as rock surrogate. This material was particularly well suited for sampling as it provided enough area for biofilm growth and furthermore enabled a fast preparation of samples as biofilm could easily be scratched off. Flumes were fed with 6 l stream water, which was pumped from a canister by an EDEN 135 Eden WaterParadise 57197 aquarium pump, passed the flumes and finally returned back to the canister via a 3 m plastic pipe. Constant water volume within each flume was around 3 l per recirculation. Recirculation was continuously running over 5-7 days till the next water exchange.

To achieve constant light conditions for all flumes, the room was first darkened by covering the windows with protective sheets. Light was provided by fluorescent daylight lamps (Philips, 36 W), which were set on a timer in a 12 hours interval (12/12 h light / dark cycle) (*Table 2*). For the whole

experiment, a total of eight daylight lamps (120 cm) were used and radiation was controlled with a light data logger (Hobo = Onset computer Corporation). Mean water discharge within all flumes was around 50 ml/s. Flow velocity was measured by injecting a certain amount of NaCl at the inlet of all flumes and measuring changes in conductivity at the outlet. Flumes were kept constant at room temperature (*Table 2*).

3.3 Study design and experimental setup

This experiment comprised a reference, a medium and high nutrient enrichment group á five flumes. Water was taken from the "Lunzer Seebach" which showed– due to its oligotrophic conditions and constant water temperature – optimal preconditions for this nutrient enrichment experiment. The experiment lasted for over 10 weeks and was divided in a colonization phase (1), an enrichment phase (2) and a recovery phase (3).

(1) During the **colonization phase** (1st of September - 2nd of October 2015), flumes were fed with unenriched stream water. Water was exchanged every second day. Since pre-cleaned gravel was used as substrate, colonization of benthic algae and bacteria within all flumes was additionally stimulated by the insertion of pre-colonized tiles (15.7 cm^2) which had been colonized in the field over one month.

(2) The **enrichment phase** lasted over four weeks, starting on 2nd of October and ending on 30th of October. Once a week, 4x30 l canisters were refilled with fresh stream water from the "Lunzer Seebach". Whereas the reference group was fed with unenriched water, remaining canisters were then - depending on the particular treatment - enriched with inorganic nitrogen added as sodium nitrate, phosphorous as potassium hydroxide phosphate and carbon as ammonium acetate (*Table 1*). To monitor nutrient content and uptake, water samples were taken directly before and after re-filling and in the first two weeks additionally two days after enrichment. Thereby it could be seen, that phosphorous was immediately taken up, either through assimilation or P-storage within the sediment. This led to the decision of an additional phosphorous enrichment during week three and four.

Table 1: Concentrations of added nutrients were chosen on the basis of areas with moderate pastoral economy (moderate) and areas of intensive arable farming (high).

	Ammonium acetate **[CH_3COONH_4]**	***Sodium nitrate*** **[$NaNO_{3]}$**	***Potassium hydroxide phosphate*** **[KH2PO4*2H2O]**
Control	0	0	0
Moderate	3 mg/l	3 mg/l	0.04 mg/l (+ 4 ml of 100 mg/l)
High	6 mg/l	6.5 mg/l	0.15 mg/l (+10 ml of 100 mg/l

(3) Within the **recovery phase** all flumes were fed with unenriched stream water for further two weeks.

Table 2: Mean radiation (lux) and temperature (°C) + SD of control, moderate and high enriched flumes during the experiment.

	Colonization		***Enrichment***		***Recovery***	
	Rad (lux)	**°C**	**Rad (lux)**	**°C**	**Rad (lux)**	**°C**
Control	407 ± 108	18 ± 0,7	393 ± 148	14 ± 0,3	445 ± 172	13 ± 0,8
Moderate	386 ± 72	18 ± 0,3	378 ± 88	14 ± 0,1	425 ± 132	12 ± 0,2
High	445 ± 35	18 ± 0,1	330 ± 52	14 ± 0,1	401 ± 67	12 ± 0,3

3.4 Sampling schedule

Effects of continuous nutrient enrichment were investigated on both substrate types. Flumes were divided into 12 sections whereas three samples (Table 4) were taken out of each section which is a total of 15 replicates per sampling date and substratum. Sampling was done at the end of the colonization phase, weekly during the enrichment phase and once at the end of the recovery phase (Table 3). As sample preparation was very time consuming and some parameter had to be analyzed quickly, sampling of the two substrate types was done on consecutive days. Nevertheless, samples of both substrates were respectively taken from the same section which was chosen randomly.

Table 3: The table below provides an overview of the duration of all particular phases within the experiment, nutrient addition and sampling dates (blue = epilithic biofilm sampling; green = epipsammic biofilm sampling) as well as all analyzed parameters in the certain phases. Chlorophyll-a, bacterial abundance and enzymatic activity were analyzed on both substrate types and during all three phases. Nutrient uptake was analyzed via short term nutrient addition at the end of the enrichment phase and at the end of the recovery phase. Figure 2 shows a schematic overview over of the experiment.

Phase	Colonization phase	Enrichment phase	Recovery phase
Labeling	c	e1- e4	r2
Date	01.09 - 02.10	02.10 - 30.10	30.10 - 13.11
Nutrient addition	-	d1, d8, d15, d22	-
Sampling days	d30, d31	d6, d7, d13, d14, d20, d21, d27,d28	d13, d14
Parameters	Chlorophyll-a, bacterial abundance, enzymatic activity	Chlorophyll-a, bacterial abundance, enzymatic activity, nutrient uptake (d28)	Chlorophyll-a, bacterial abundance, enzymatic activity, nutrient uptake (d14)

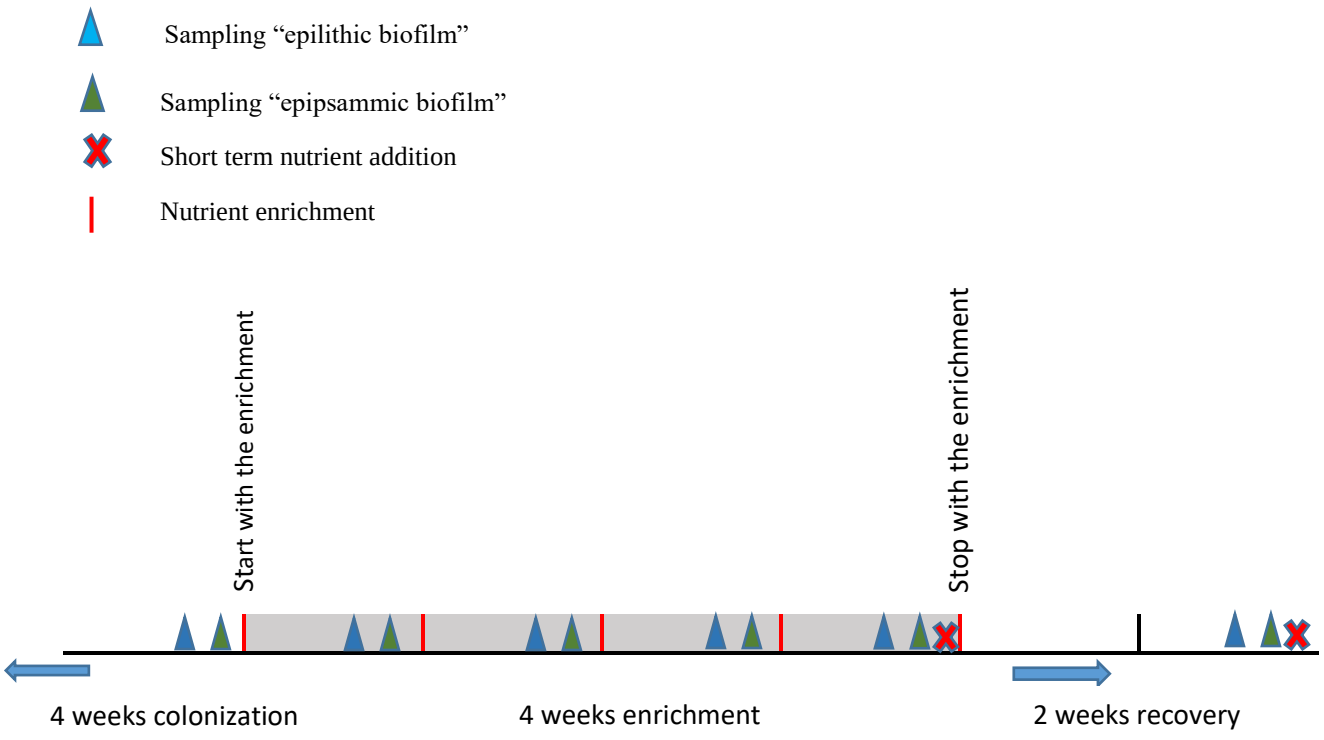

Figure 2: Schematic overview of the experiment.

3.5. Sample preparation

(1) Biofilm analyses on glass substrata, all three Epilithic (replicates) within a section were transported to the lab, where the biofilm was scratched off from each slide into a separate 50 ml plastic tube and afterwards filled up with 10 ml autoclaved water. This suspension was then split on smaller tubes for chlorophyll-a, enzymatic activity and bacterial density. **(2) For the analyzation of biofilm on gravel substrate**, three replicates were taken by using a cylinder (3 cm diameter, 1.5 cm depth). Gravel was directly transferred into a 50 ml plastic tube and supernatant was removed carefully. Samples were first weighed in total before they were split up on smaller plastic tubes and filled up with autoclaved water.

Hydrolytic enzymes were directly measured after transporting samples to the lab. For chlorophyll-a analyzation, subsample were filtered through a 47-mm-diameter fiberglass filter (GF/C Whatman) put in labeled alu-sheets and kept frozen until analysis. The suspension for bacterial abundance was fixed with 160 µL 37% formol and then stored at 4°C. Detailed method description below.

Table 4: Per sampling day, three replicates were taken out of a section. Suspension of scratched of biofilm mixed with autoclaved water was first homogenized and then split up in subsamples for the particular parameter. Gravel samples (epipsammic) were split to subsamples and filled up with autoclaved water.

	Replicates per flume	Chlorophyll a	Bacterial abundance	Enzymatic activity
Epilithic	3	5 ml	3 ml	2 ml
Gravel	3	5g (+10 ml a.w)	3g (+10 ml a.w)	1g (+10 ml a.w)

Chlorophyll-a

For the extraction, frozen filters were first cut into small pieces, put into glass vials and extracted with 6-10 ml 90% acetone. Afterwards, the suspension was sonicated (Branson Digital Sonifier, Sonifier W 250D; amplitude: 30%, time: 30 sec, interval: 1 sec/1sec) and stored light-protected for 12 - 24 hours at a temperature of 4°C. Sonication is a necessary step to guarantee complete chlorophyll-a extraction. After 12 - 24 hours, samples were centrifuged at 2500 rpm for 10 minutes (Hettich Zentrifugen, Rotanta 460R). As samples were highly concentrated, samples were diluted by 1:5, 1:10 or 1:20 depending on the colouring before fluorescence measurement. The samples were measured in a 1 cm quartz cuvette with a fluorometer (HITACHI, F-7000 Fluorescence Spectrophotometer). An acetone blank was measured first which was then abstracted from the

measured chlorophyll-a values. Samples were then analyzed by transferring 1 ml of the supernatant carefully into a 1cm quartz cuvette. Pheophytin was measured after acidifying samples with 0,1 ml of a 0,1 N HCl and 90 seconds waiting time (Steinman et al., 2017).

Bacterial abundance

For counting, each sample was mixed with 10 ml of a 0.22 µm sodium pyrophosphate ($Na_4P_2O_7$) solution (10 mM) and a Tween 80 (10 %), which is used for the separation of bacteria attached to sediment. Subsequently samples were sonicated (Bandelin Sonopuls HD2200) for 1 minute (14 % amplitude, pulse on 1 sec, pause 1 sec) to avoid aggregation of the material. Afterwards, samples were centrifuged, diluted (ranging from 1:10 - 1:400) and stained with SYBR Green II. The stained samples were incubated in the dark for 10 minutes. The whole content of the tube was then filtered on a black membrane filter (0,2 µm, Millipore, GTBP02500), transferred to a glass slide and embedded in paraffin oil (Duhamel and Jacquet, 2006). Bacterial abundance per milliliter was counted directly afterwards under a fluorescence microscope (Zeiss Imager Z1, Filter: Zeiss 38, Objektiv: EC Plan – Neofluar 100x/1.3 oil iris).

Enzymatic activity

This experiment focused on the activity of four different types of hydrolytic enzymes (ß-glucosidase, ß-xylosidase, leucine-aminopeptidase and phosphatase. For the measurement, 200 µl of each subsample was mixed with 50 µl of substrate (samples: 2 analytical repliates). Methylumbelliferyl (MUF)-linked substrate (4-Methyllumbelliferyl β-D glucopyranoside; 4-Methyllumbelliferyl β-D xylopyranoside; 4-Methylumbelliferyl phosphate) was used for glucosidase, xylosidase and phosphatase, while aminomethyl-coumarin (AMC)-linked substrate (L-Leucine-7-amido-4-methylcoumarin hydrochloride) was used to analyze amino-peptidase. Standard rows and a homogenate mix were also pipetted on the plate and enriched with substrate. Plates were carefully mixed through and inserted into the microplate reader (Thermofischer Scientific, Varioskan Flash). The addition of artificial fluorometric substrate led to substrate hydrolysis causing a fluorescence signal, which could be detected by the microplate reader (software: SkanIt RE for Varioskan Flash 2.4.5. ex: 365 nm, em: 450 nm). After measuring, plates were incubated in a climate chamber under dark conditions. After approximately 60 minutes, plates were measured again to calculate the actual enzyme activity (Jackson et al., 2013)

Nutrient uptake

Nutrient uptake was analyzed by injecting 20 ml of a 100 mg/l P-PO4 solution to all flumes. After one hour, samples were taken in one-hour intervals for further 5 hours. The samples were filtered through GF/F filter in acid-washed glass vials and P-PO4 concentration was analyzed by a continuous flow analyzer (CFA, Systema Analytical Technology) (Munn and Meyer, 1990; Payn et al., 2005)

Decomposition

Pre-weighed wooden sticks, which were dried at a temperature were placed within each flume as an additional refractory material (Zealand et al., 2009).

3.6 Calculation and Statistics

Algal biomass

Each sample was measured three times and averaged. Furthermore, the acetone blank was subtracted. Chlorophyll-a concentration was calculated with following formulas in consideration of pheopigments, extrapolated to the total volume / sampled area.

Chla (0 – 200 µg/l):	$\frac{(0.9185 * \left(\frac{\mathrm{r}}{\mathrm{r}-1}\right) * (Rb - Ra) + 1.9217) * Vol.extrak\ (l)}{\mathrm{Vol.\,sample}\ [l, cm2, g]}$
Chla (200 – 1000 µg/l):	$\frac{(1.1522 * \left(\frac{\mathrm{r}}{\mathrm{r}-1}\right) * (Rb - Ra) - 67.724) * Vol.extrak\ (L)}{\mathrm{Vol.\,sample}\ [l, cm2, g]}$
Chla (1000– 1900 µg/l):	$\frac{\left(1.7347 * \left(\frac{\mathrm{r}}{\mathrm{r}-1}\right) * (Rb - Ra) - 655.95\right) * Vol.extrak\ (l)}{\mathrm{Vol.\,sample}\ [l, cm2, g]}$

r (acidification factor)= 18,9

Rb = value before acidification

Ra = value after acidification

Bacterial abundance

Bacterial abundance was calculated via the formula: $\frac{AF}{AR} * b * df$ *(AF … area of filter, AR … area of raster, b … average of bacterial count of 20 rasters, df … dilution factor)*. Evaluated bacterial abundance per ml was then extrapolated to the sampling area.

Enzymatic activity

As mentioned in 3.4.3, enzymes were measured twice with an interval of 60 minutes. Concentrations for each sample were calculated separately for the first and the second run via the corresponding standard row (MUF/AMC). Then calculated concentrations of the first run were subtracted from the calculated concentrations of the second run. For the final calculation of enzymatic activity rate, this result had to be multiplied with the dilution factor, extrapolated to the volume of the original sample and divided by the corresponding area / weight of the substrate. The rate per hour was finally determined in consideration of the actual interval between first and second measurement.

Statistical analysis

IBM SPSS 21 was used for all kinds of statistical analysis within this work. Measured values of all parameters were illustrated within a boxplot showing the particular sampling week but also the particular treatment. This illustration allowed to give information about the temporal development within one treatment but also about the differences between all treatments (control, moderate and high enrichment) over a period of 7 weeks. As sample size was rather small and data was not normally distributed, all hypothesis of investigated parameters were tested with a median test to minimize the effects of outliers. If the median test showed any significant differences, a pairwise comparison based on a chi^2 test was made in order to detect which treatments or, referring to the temporal development, which weeks significantly differed from each other. Although median test has noticeably lower power, the results showed a clear pattern.

4. Results

4.1 Nutrient concentration

4.1.1 N-NH4

Figure 3 shows the mean ammonia concentrations (µg/l) within all experimental groups for the whole sampling phase. Control group revealed a mean of 20.8 ±3.56 during colonization phase, 21.9 ±7.98 during enrichment phase and 10.8 ±2.78 during recovery phase. Moderate enrichment had a slightly lower initial concentration during colonization phase (14.8 µg/l ±2.68) but a relatively high mean of 995.4 ± 103.2 during enrichment phase, which cannot even be found in the "Weinviertel", one of the most productive agricultural areas in Austria. Ammonia during recovery phase again was at a very low level of 10.8 ±6.06. High-enriched group showed a mean ammonia concentration of 19.6 ±2.44 during colonization and reached an oversaturation with 2175.75 ± 309.19 during enrichment phase, followed by 31.6 ± 49.45 during recovery phase.

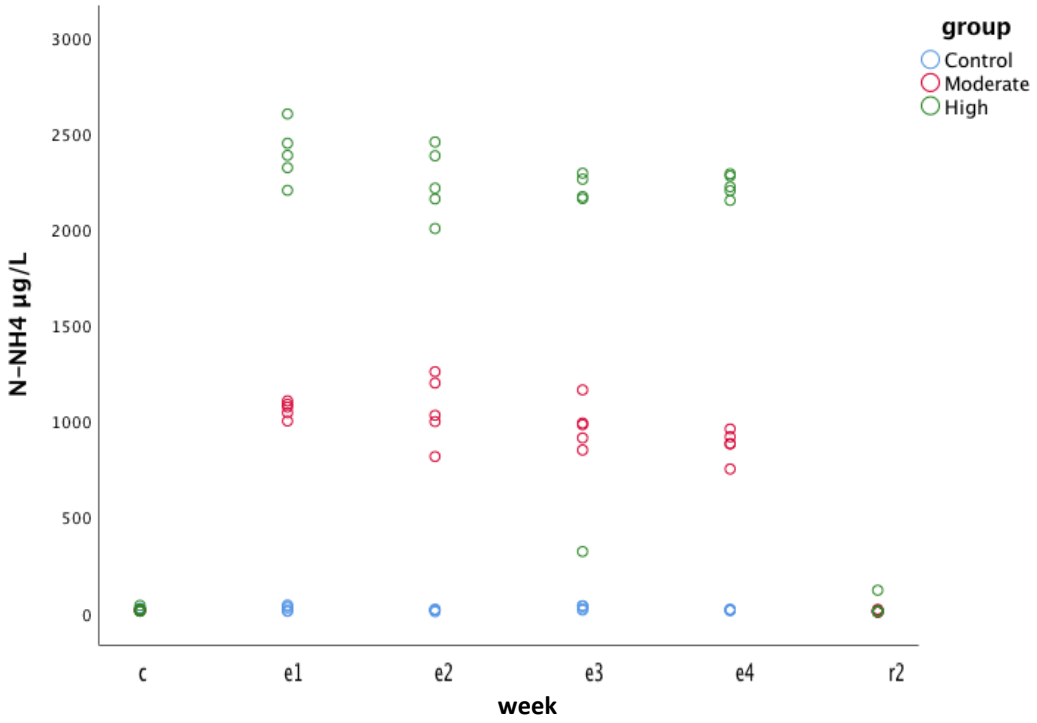

Figure 3: N-NH4 concentrations in control group, moderate and high enriched flumes throughout the experiment.

4.1.2 N-NO3

Figure 4 represents the mean nitrate concentrations (µg/l) within all experimental groups for the whole sampling phase. Control group revealed a mean initial concentration of 440.33 ± 184.03, 541.35 ± 118.79 during nutrient enriched phase and 266.20 ± 106.37 during recovery phase. Moderate enrichment showed an average nitrate concentration of 410.06 ± 102.23 during colonization phase, 3567.15 ± 241.14 during enrichment phase and 157.20 ± 115.78 during recovery phase. High-enriched group had a mean nitrate concentration of 402.96 ± 90.83 during colonization, 7106.90 ± 880.74 during enrichment phase and 250.00 ± 419.80 during recovery phase. Nitrate concentrations within enriched flumes are similar to eutrophic streams in non-intensive agricultural used areas.

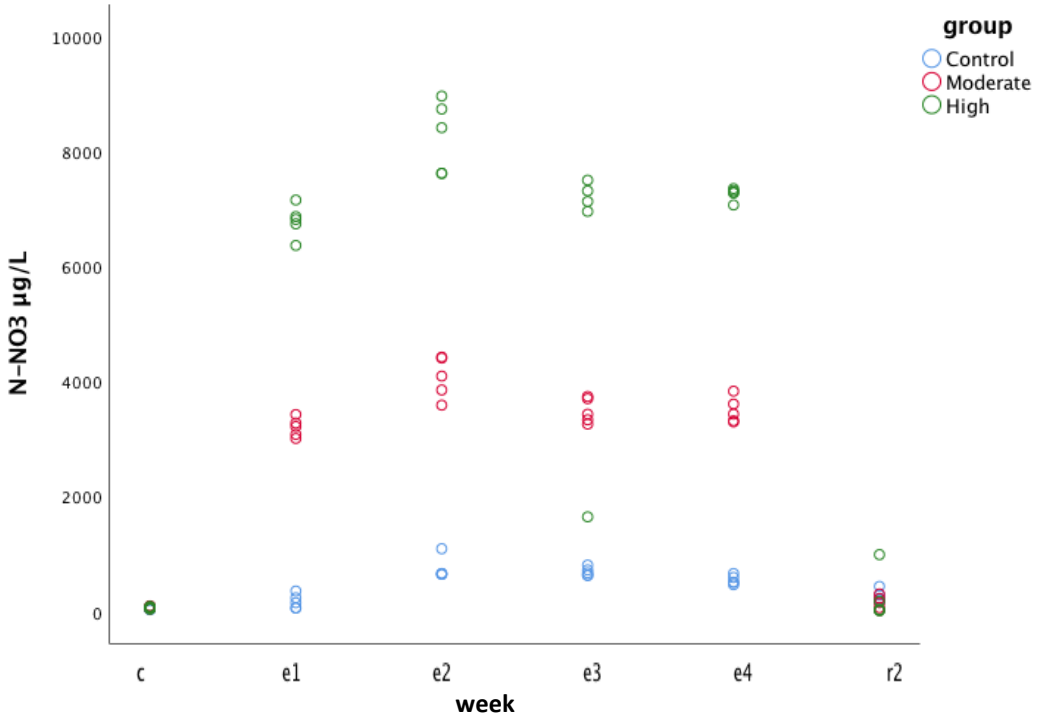

Figure 4: N-NO^3 concentrations in control group, moderate and high enriched flumes throughout the experiment.

4.1.3 P-PO4

Figure 5 shows the mean phosphate concentrations (µg/l) within all experimental groups for the whole sampling phase. Control group revealed a mean of 1.4 ± 2.07 during colonization phase, 0.05 ± 0.12 during enrichment phase and 0.2 ± 0.45 during recovery phase. Average phosphate concentration of moderate enrichment was ~0 during colonization phase, 8.8 ± 3.06 during enrichment phase, which is similar to concentrations in eu-polytrophic streams in non-intensive agricultural used areas and ~0 again during recovery. In contrast to control and moderate enriched group, the high-enriched group already had a relatively high mean phosphate concentration of 9.40 ± 9.78 during colonization followed by 61.65 ±10.01 during enrichment phase representing weak eu-polytrophic conditions followed by phosphate poor conditions within recovery phase.

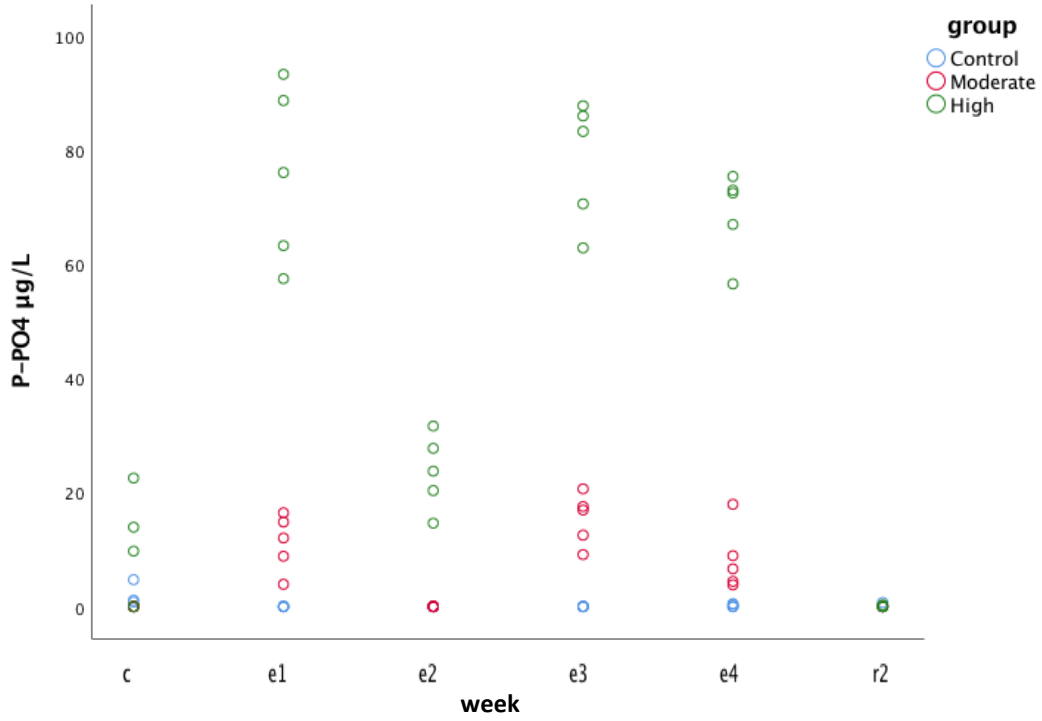

Figure 5: P-PO4 concentrations in control group, moderate and high enriched flumes throughout the experiment. In e3 and e4, nutrient enriched flumes were additionally enriched with a phosphate solution.

4.1.4 DOC

Measured mean of DOC in colonization phase was around 1.6 mg/l within each experimental group. Although measured DOC in control group stayed on a very similar level throughout the whole experiment it could be observed, that from e1 to e3 concentrations after refilling slightly increased. During enrichment phase, DOC in moderate-enriched group (mean of ~4.2 mg/l ± 0.69) as well as high-enriched group (mean of ~6.7 mg/l ± 0.98) strongly decreased within the particular sampling weeks. Differences between the left bar (DOC concentration after enrichment at the beginning of the week) and the right bar (DOC concentration at the end of the week) are slightly higher in week "e4".

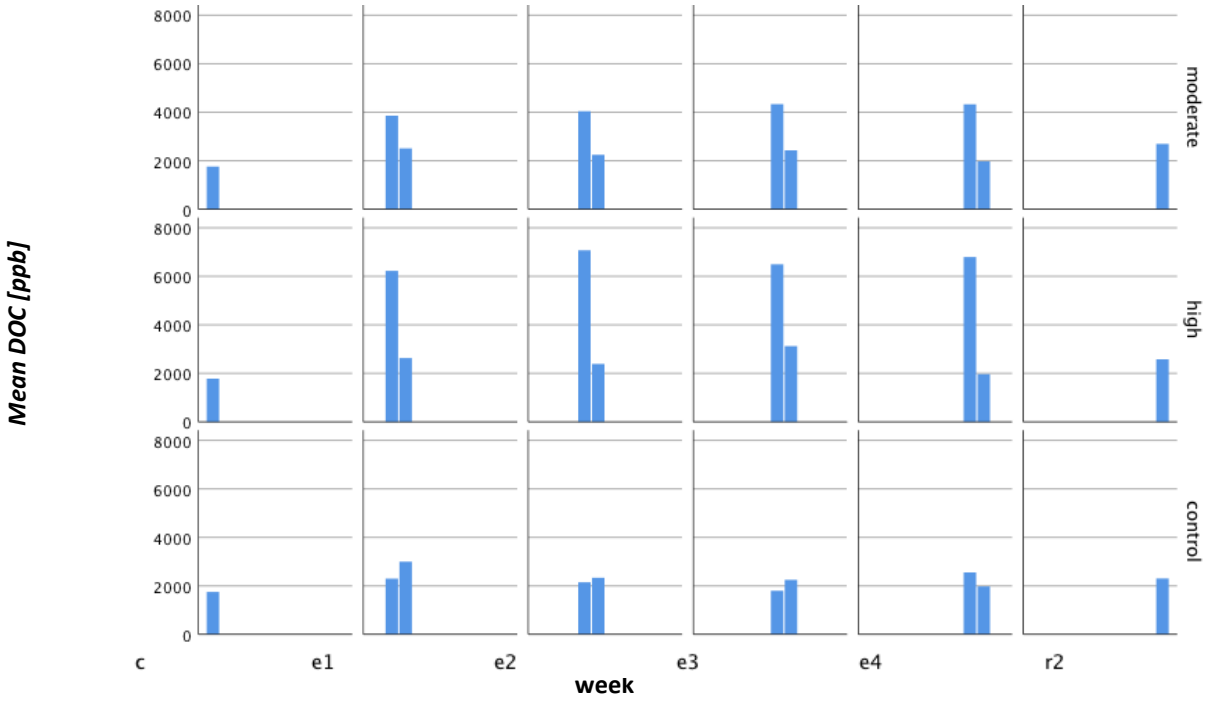

Figure 6: Mean DOC concentrations throughout the experiment (column: weeks; rows: experimental group). Whereas DOC in colonization and recovery phase was just measured once, the two bars shown in each week of enrichment phase display concentrations directly after enrichment and directly before the next enrichment.

4.2 Chlorophyll-a

4.2.1 Epilithic biofilm

A significant positive effect of nutrient enrichment on chlorophyll-a could be examined on epilithic biofilms (Figure 7). Whereas the colonization phase was characterized by similar initial concentrations, the first nutrient enrichment (e1) already caused higher chlorophyll-a concentrations in the high-nutrient enriched group. Applied median tests (Table 5) revealed that up to week 4, nutrient enrichment clearly supported the growth of epilithic biofilms, which could be seen by the strong increase within treated groups. But also the control group slightly increased up to week 4 implying that colonization was not yet completed (Table 6) However, measured chlorophyll-a in treated groups clearly exceeded biomass production of the control group, but also within nutrient treated groups differences were significant. During week 4, maximum chlorophyll-a concentrations were reached in high-enriched flumes with a mean of ~1200 µg/cm^2 followed by moderate enriched flumes with a mean around 800 µg/cm^2. During recovery phase, the high-enriched group showed a reduction of biomass very similar to moderate enrichment, but both treated groups were still significantly higher than the control group. The temporal development within the particular treatment revealed, that despite the reduction of biomass within recovery, end-chlorophyll-a concentrations were still significantly higher than initial concentrations.

4.2.2 Epipsammic biofilm

In contrary, the epipsammic biofilm (Figure 8) showed smaller enrichment effects than the epilithic biofilm. Whereas conspicuous effects between treated groups and control group could first be proven in week 2 of the enrichment phase, nutrient enriched flumes did not show any significant differences amongst each other. Also further development did not show any noteworthy changes between (Table 5) and within the particular groups (Table 6). Despite the two weeks of non-nutrient-enrichment, effects of recovery were not visible. Chlorophyll-a concentrations of treated groups still exceeded concentrations of control group, but also within the particular groups, concentrations did not decrease to initial concentrations of colonization phase. Results of the applied median test and pairwise comparison can be seen in Table 6.

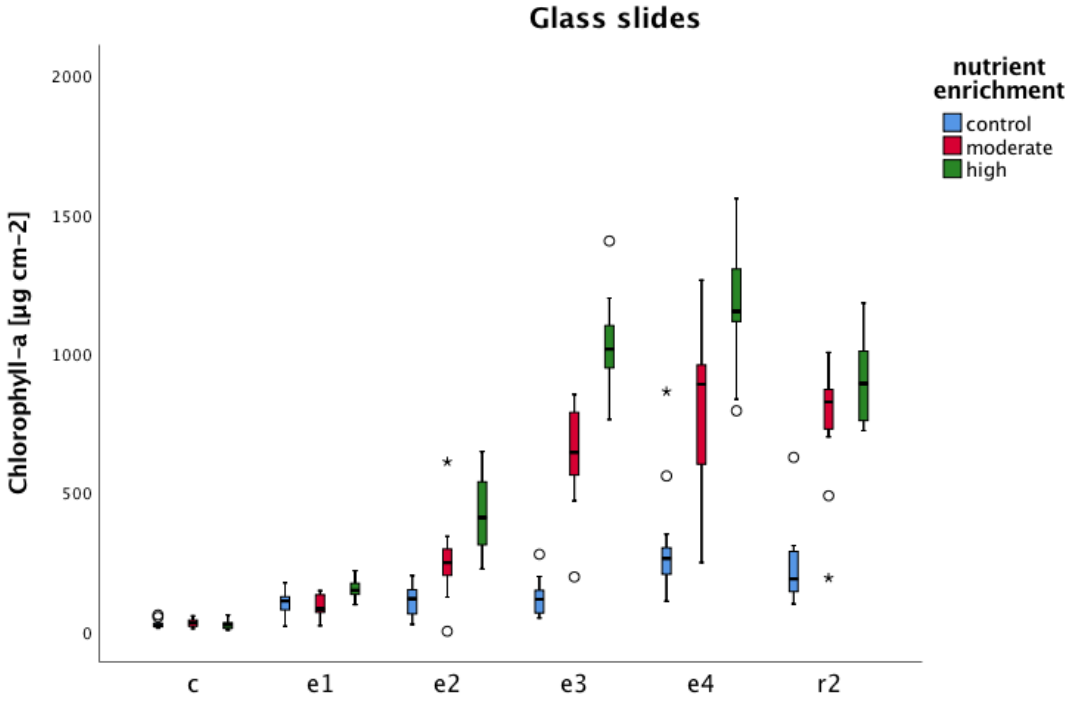

Figure 7: Chlorophyll-a concentrations of epilithic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date.

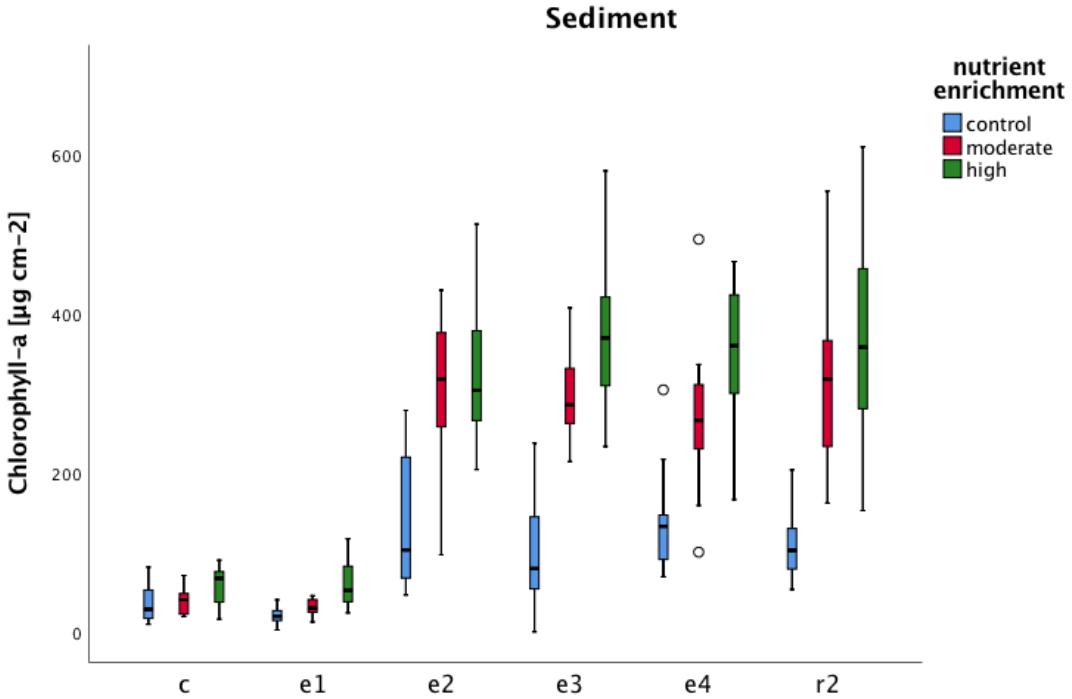

Figure 8: Chlorophyll-a concentrations of epipsammic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

Table 5: Effects of nutrient enrichment on chlorophyll-a in epilithic and epipsammic biofilm were tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). Pairwise comparison was made *between control group (C), moderate (M) and high (H) enrichment.*

Epilithic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 0.95$	2	0.62	-
e1	$Chi^2 = 9.07$	2	0.01	C-H; C-M
e2	$Chi^2 = 24.53$	2	0.00	C-M; C-H; M-H
e3	$Chi^2 = 24.00$	2	0.00	C-M; C-H; M-H
e4	$Chi^2 = 20.42$	2	0.00	C-M; C-H; M-H
r2	$Chi^2 = 19.82$	2	0.00	C-M. C-H

Epipsammic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 2.15$	2	0.34	-
e1	$Chi^2 = 14.98$	2	0.01	C-H
e2	$Chi^2 = 9.60$	2	0.01	C-M. C-H
e3	$Chi^2 = 22.67$	2	0.00	C-M; C-H; M-H
e4	$Chi^2 = 15.54$	2	0.00	C-M; C-H; M-H
r2	$Chi^2 = 21.07$	2	0.00	C-M. C-H

Table 6: Effects of time on chlorophyll-a concentrations tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). The table below shows the pairwise comparison *between particular weeks of the experiment (c = colonization; e1 – e4 = nutrient enrichment; r2 = recovery phase).* Significant values are highlighted.

Epilithic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 31,02$	5	0.00
Moderate	$Chi^2 = 67,01$	5	0.00
High	$Chi^2 = 76,14$	5	0.00

Epipsammic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 78,32$	5	0.00
Moderate	$Chi^2 = 34,36$	5	0.00
High	$Chi^2 = 33,11$	5	0,00

Epilithic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Moderate	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
High	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	1.00	1.00	**0.00**	1.00	0.00	0.00	0.75
Moderate	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	1.00	1.00
High	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.03**	**0.02**

Epipsammic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	1.00	0.07	0.12	**0.00**	**0.02**
Moderate	1.00	**0.00**	**0.00**	**0.00**	**0.00**
High	1.00	**0.02**	**0.02**	**0.03**	**0.00**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.00**	**0.00**	**0.00**	1.00	1.00	1.00	1.00
Moderate	**0.00**	**0.00**	**0.00**	1.00	0.93	1.00	0.87
High	**0.00**	**0.00**	**0.00**	1.00	1.00	1.00	1.00

4.3 Bacteria

4.3.1 Epilithic biofilm

The analysis in epilithic biofilm (Figure 9) also revealed effects of nutrient enrichment on bacterial abundance. First effects appeared in week 2, where enriched flumes significantly differed from control group (Table 7). However, further development showed, that while bacterial abundance in week 4 was highest in high-enriched flumes, control group also increased and exceeded moderate enriched flumes (Table 8). Control group but also moderate enriched group significantly differed to high-enriched flumes. Focussing on the temporal development, bacterial abundance showed an increase over time, regardless of the group. As expected, the steepest increase over time could be detected within high enriched flumes. Recovery phase led to an approximation of the different groups and did not reveal any significant differences. Also the temporal development within the particular groups was not significant. Nevertheless, by comparing colonization phase and recovery phase it could be detected, that bacterial abundance was still significantly higher in the recovery phase.

4.3.2 Epipsammic biofilm

Epipsammic biofilm (Figure 10) was characterized by a general decrease of all groups between colonization phase and week 2 of nutrient enrichment phase (Table 8). Temporal development between e2 and e4 showed that nutrient treated groups significantly increased again. However, differences between the particular groups were not significant at the end of enrichment phase (Table 7). Within two weeks of recovery phase, changes neither within nor between the groups were found. However, pairwise comparison between colonization and recovery phase showed significant differences, but in contrary to the epilithic biofilm, bacterial abundance within recovery phase was lower than in colonization phase, regardless of the experimental group.

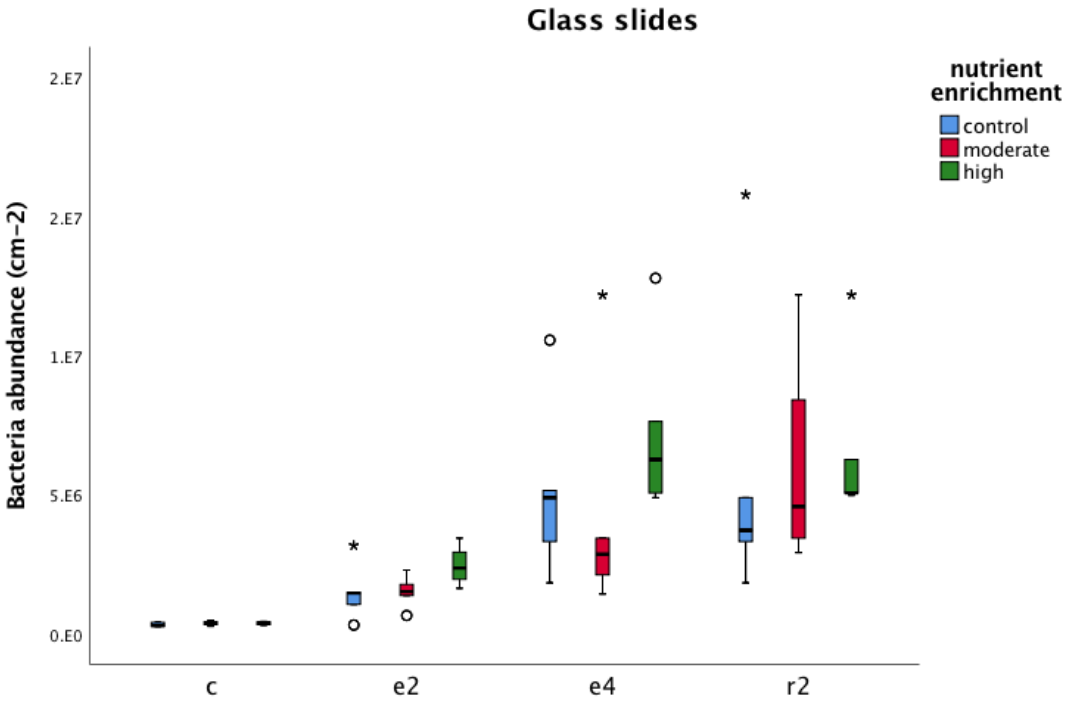

Figure 9: Bacterial abundance of epilithic biofilm showing control group, moderate and high nutrient enrichment in c, e2, e4 and r2. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date.

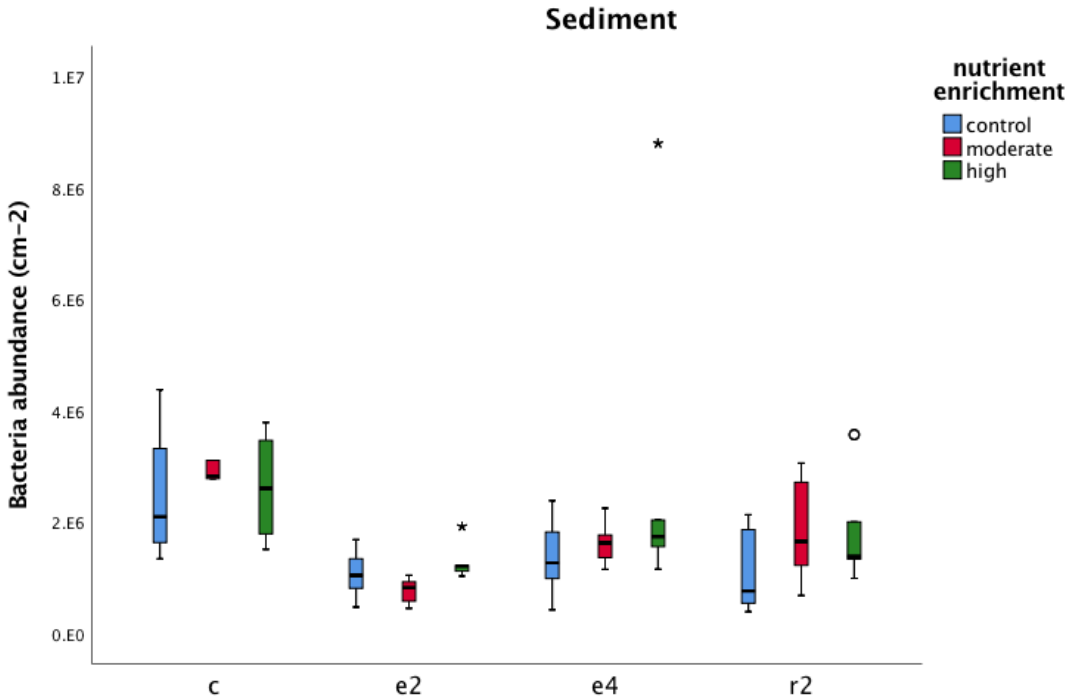

Figure 10: Bacterial abundance of epipsammic biofilm showing control group, moderate and high nutrient enrichment in c, e2, e4, and r2. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n=45 per treatment and date).

Table 7: Effects of nutrient enrichment on bacterial abundance in epilithic and epipsammic biofilm were tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). Pairwise comparison was made between control group (C), moderate (M) and high (H) enrichment.

Epilithic

	Test statistic	**df**	**Asymptotic sig.**	**Pairwise Comparison**
c	$Chi^2 = 0.74$	2	0.64	-
e2	$Chi^2 = 18.00$	2	0,00	C-H, M-H
e4	$Chi^2 = 11.25$	2	0.00	M-H
r2	$Chi^2 = 11.25$	2	0.00	C-H

Epipsammic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 6.74$	2	0.03	C-H, M-H
e2	$Chi^2 = 11.25$	2	0,00	M-H
e4	$Chi^2 = 1.61$	2	0.45	-
r2	$Chi^2 = 3.75$	2	0.15	-

Table 8: Effects of time on bacterial abundance tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). The table below shows the pairwise comparison between particular weeks of the experiment. This parameter was just analyzed in c, e2, e4 and r2. Significant values are highlighted.

Epilithic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 25.2$	3	0.00
Moderate	$Chi^2 = 32.93$	3	0.00
High	$Chi^2 = 44.4$	3	0.00

Epipsammic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 18.45$	3	0.00
Moderate	$Chi^2 = 25.20$	3	0.00
High	$Chi^2 = 9.47$	3	0.03

Epilithic

	c – e2	**c – e4**	**c – r2**
Control	**0.00**	**0.00**	**0.00**
Moderate	**0.00**	**0.00**	**0.00**
High	**0.00**	**0.00**	**0.00**

	e2 – e4	**e2 – r2**	**e4 – r2**
Control	**0.01**	**0.01**	1.00
Moderate	**0.01**	**0.00**	0.15
High	**0.00**	**0.00**	1.00

Epipsammic

	c – e2	**c – e4**	**c – r2**
Control	**0.03**	**0.03**	**0.03**
Moderate	**0.00**	**0.00**	**0.00**
High	**0.03**	**0.03**	**0.03**

	e2 – e4	**e2 – r2**	**e4 – r2**
Control	1.00	1.00	1.00
Moderate	**0.00**	**0.01**	1.00
High	**0.01**	**0.01**	1.00

4.4 Activity of alkaline Phosphatase (APA)

4.4.1 Epilithic biofilm

Though both sediment types clearly reacted to nutrient enrichment during the whole experiment, APA in epilithic biofilms (Figure 11) were 10 times as high as in epipsammic biofilms (Figure 12). APA in colonization phase already showed differences amongst each other. Nevertheless, effects due to nutrient addition between experimental groups became very dominant during the four weeks of enrichment phase, where phosphatase activities were highest in moderate enriched flumes on most of the sampling dates and significantly differed from control flumes (Table 9, Table 10). Differences to high-enriched flumes were not significant during the enrichment phase. Also by considering the temporal development within all groups it could be seen, that differences of measured APA were more striking with increasing nutrient addition. Solely between e3 and e4, detected concentrations within all flumes almost stayed on the same level and did not show any significant changes (Table 10). After two weeks of recovery, the release of phosphatase strongly decreased especially in enrichment groups, but released phosphatase was still higher than in colonization phase. However, in contrary to previous measurements, the high nutrient enrichment then exceeded moderate enrichment. All groups significantly differed between each other (Table 9).

4.4.2 Epipsammic biofilm

In epipsammic biofilms (Figure 12), nutrient enrichment also positively affected the phosphatase activity. The temporal development of epipsammic biofilm followed a similar pattern as in epilithic biofilm towards e2, but in contrary to epilithic biofilm, epipsammic biofilm strongly decreased in e3 (Table 10). Referring to differences within the groups it could be seen, that moderate enrichment in epipsammic biofilm remarkably increased the release of phosphatase in e2. Also e3 was characterized by highest mean phosphatase in moderate enrichment but differences were just significant to high enriched flumes. Between e3 and e4, neither nutrient treated groups nor control group showed prominent changes (Table 9). Within the two weeks of recovery, all groups slightly increased but only changes within control group and moderate nutrient enrichment were significant (Table 10). Considering differences between the groups, recovery phase also led to an approximation between control and moderate enriched groups but concentrations in high enriched flumes were still significantly higher (Table 9).

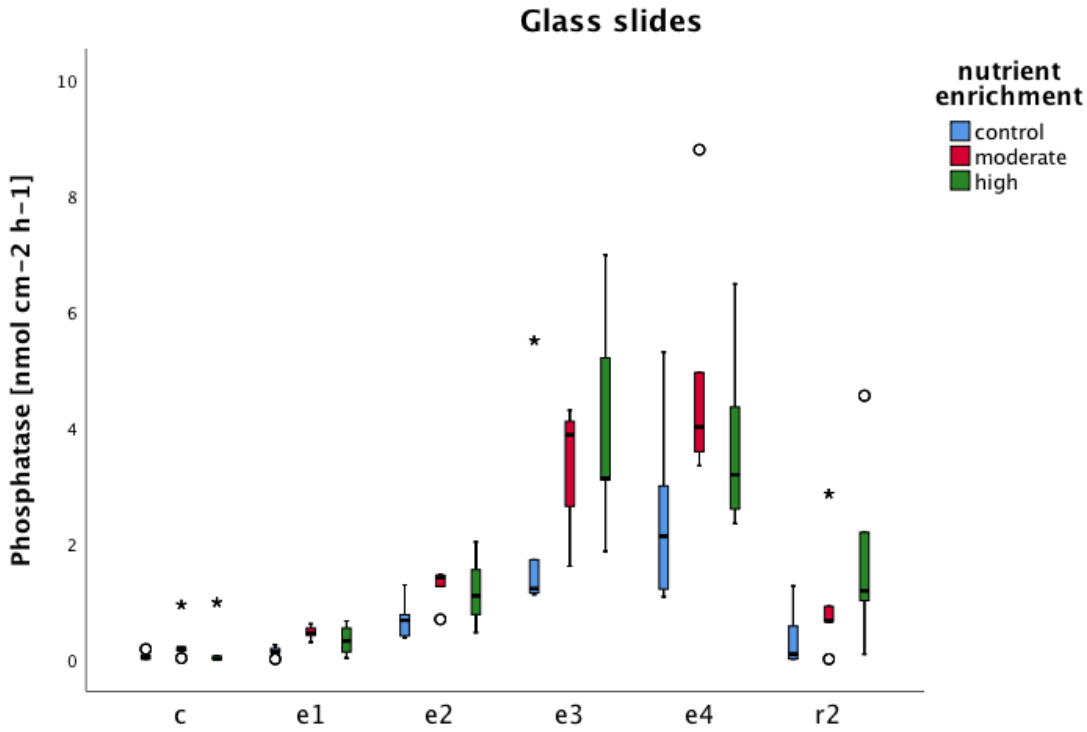

Figure 11: Phosphatase activity in epilithic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

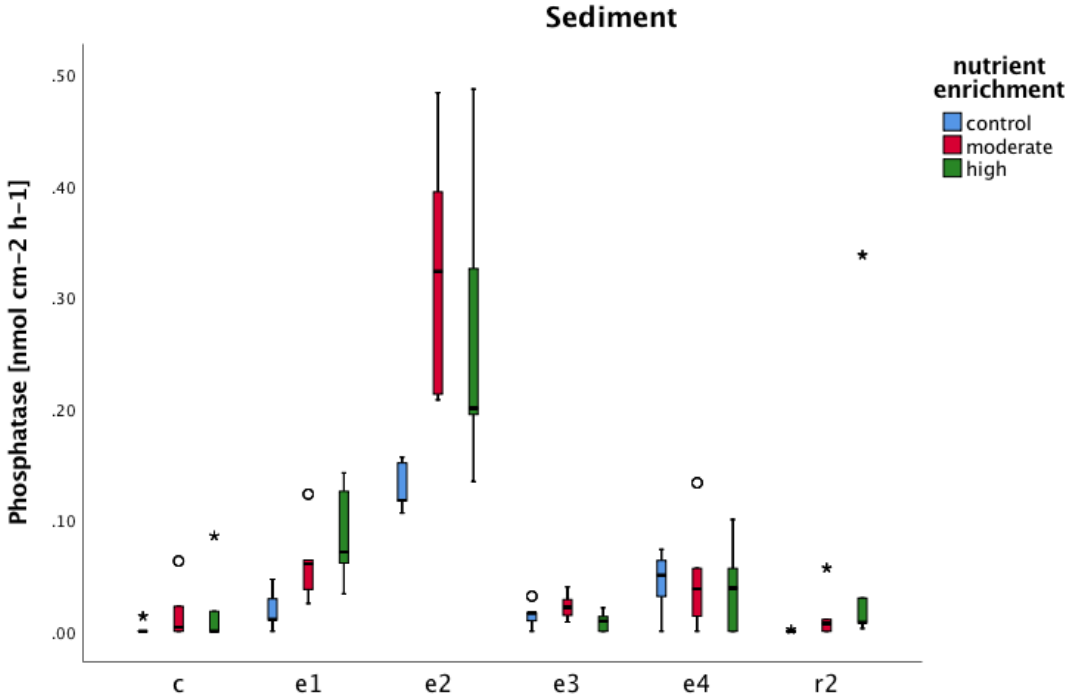

Figure 12: Phosphatase activity in epipsammic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

Table 9: Effects of nutrient enrichment on phosphatase activity in epilithic and epipsammic biofilm tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). Pairwise comparison was made between control group (C), moderate (M) and high (H) enrichment.

Epilithic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 11.25$	2	0.00	C-M, H-M
e1	$Chi^2 = 20.89$	2	0.00	C-M
e2	$Chi^2 = 11.25$	2	0.00	C-M, C-H
e3	$Chi^2 = 6.43$	2	0.04	C-M, C-H
e4	$Chi^2 = 11.25$	2	0.00	C-M
r2	$Chi^2 = 11.25$	2	0.00	C-M, C-H, M-H

Epipsammic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 5.00$	2	0.08	-
e1	$Chi^2 = 20.89$	2	0.00	C-M, C-H
e2	$Chi^2 = 30.54$	2	0.00	C-M, C-H
e3	$Chi^2 = 6.43$	2	0.04	M-H
e4	$Chi^2 = 1.61$	2	0.45	-
r2	$Chi^2 = 20.84$	2	0.00	C-H

Table 10: Effects of time on phosphatase activity tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). The table below shows the pairwise comparison between particular weeks of the experiment (c = colonization; e1 – e4 = nutrient enrichment; r2 = recovery phase). Significant values are highlighted.

Epilithic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 61.20$	5	0.00
Moderate	$Chi^2 = 70.8$	5	0.00
High	$Chi^2 = 61.2$	5	0.00

Epipsammic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 42.00$	5	0.00
Moderate	$Chi^2 = 37.20$	5	0.00
High	$Chi^2 = 56.40$	5	0,00

Epilithic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	0.02	**0.00**	**0.00**	**0.00**	1.00
Moderate	0.02	**0.02**	**0.00**	**0.00**	**0.02**
High	0.02	**0.02**	**0.00**	**0.00**	**0.02**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.00**	**0.00**	**0.00**	**0.02**	**0.02**	1.00	**0.02**
Moderate	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	1.00	**0.00**
High	**0.02**	**0.00**	**0.00**	**0.02**	**0.00**	1.00	**0.02**

Epipsammic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	**0.02**	**0.00**	**0.02**	**0.02**	1.00
Moderate	**0.02**	**0.00**	1.00	1.00	1.00
High	**0.02**	**0.00**	1.00	1.00	1.00

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.00**	1.00	**0.02**	**0.00**	**0.00**	1.00	**0.02**
Moderate	**0.00**	**0.02**	1.00	**0.00**	**0.00**	1.00	**0.02**
High	**0.02**	**0.00**	1.00	**0.00**	**0.00**	1.00	1.00

4.5 Glucosidase

4.5.1 Epilithic biofilm

Up to e3, moderate and high nutrient enrichment in epilithic biofilm had slightly higher values than in control group (Figure 13) implying, that the temporal development within treated flumes was more prominent up to this point. Nevertheless, measured glucosidase activities of control group and moderate enrichment did not differ significantly in contrast to high-enriched flumes, which significantly differed to both groups. In e4, both nutrient treated groups strongly increased their release of glucosidase and significantly exceeded control group showing highest measured values in high enrichment group. After two weeks of recovery, impacts of nutrient enrichment were still visible. Solely moderate enrichment showed a significant lowered enzyme production. Nevertheless, recovery did not lower the glucosidase production to initial concentrations. Results of pairwise comparison can be seen in Table 11.

4.5.2 Epipsammic biofilm

First effects of nutrient enrichment on epipsammic biofilm (Figure 14) could be detected in e2, where measured values of control group as well as moderate enriched flumes were significantly lower than in high-enriched flumes (Table 11). However, the temporal development of all experimental groups generally showed a decreasing trend of released glucosidase in e2 but increased again towards the end of enrichment phase, where moderate as well as high enrichment went to a very similar level (Table 12). Comparing e1 and e4 within the particular groups, overall changes are not very prominent. Nonetheless, nutrient treated groups showed a slight increase. The influence of nutrient addition on the release of glucosidase became more visible by comparing colonization phase and e4, where differences in treated groups were significantly higher (Table 12). Two weeks of recovery affected treated groups but not in a significant way. Except high-enriched group, measured concentrations in r2 went back to a very similar level as in colonization phase. Thus, solely high-enriched flumes were still significantly higher than the control group.

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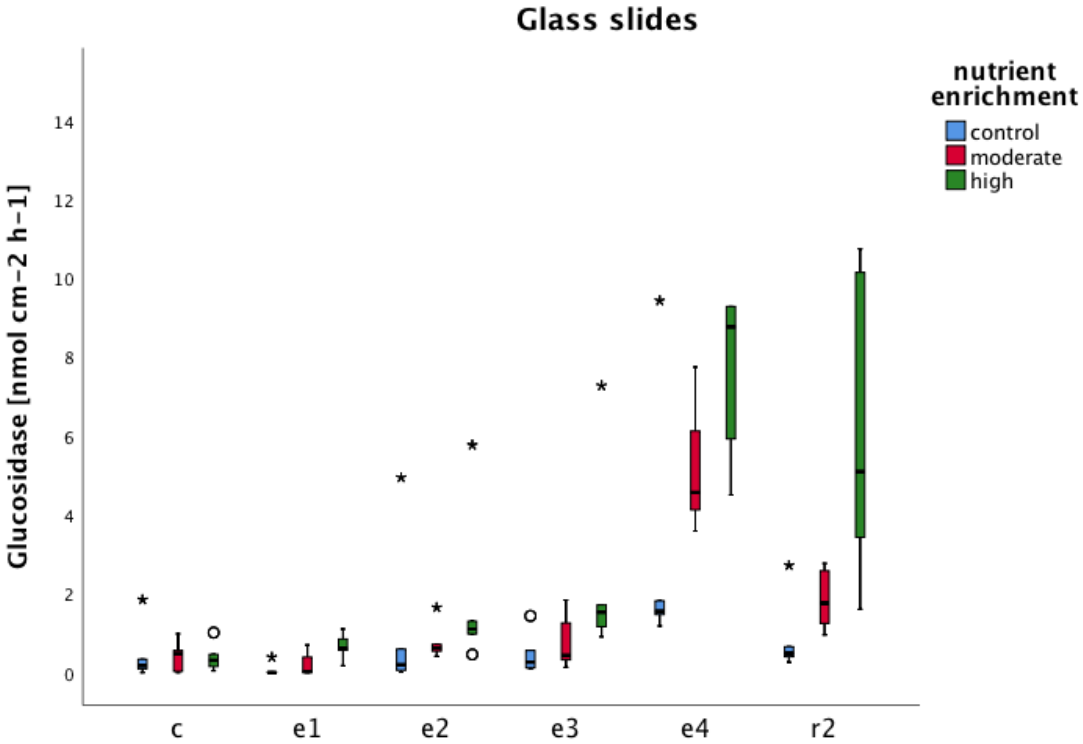

Figure 13: Glucosidase activity in epilithic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

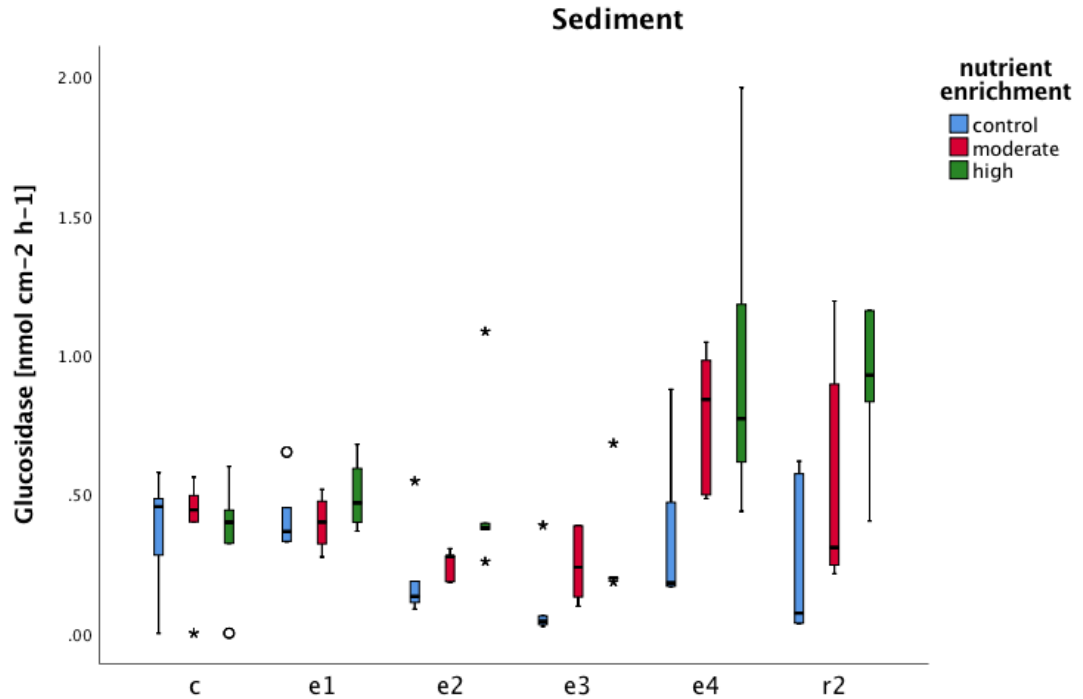

Figure 14: Glucosidase activity in epipsammic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 15 per treatment and date).

Table 11: Effects of nutrient enrichment on glucosidase activity in epilithic and epipsammic biofilm tested by median test. Significant differences are based on a Chi^2 test ($p < 0.05$). Pairwise comparison was made between control group (C), moderate (M) and high (H) enrichment.

Epilithic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	Chi^2 =1.61	2	0.50	-
e1	Chi^2 =11.25	2	0.00	C-H, M-H
e2	Chi^2 =11.25	2	0.00	C-H, M-H
e3	Chi^2 =11.25	2	0.00	C-H
e4	Chi^2 =11.25	2	0.00	C-M, C-H
r2	Chi^2 =11.25	2	0.00	C-M, C-H, M-H

Epipsammic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	Chi^2 =6.43	2	0.82	-
e1	Chi^2 =6.41	2	0.04	M-H
e2	Chi^2 =11.25	2	0.00	C-H, M-H
e3	Chi^2 =11.25	2	0.00	C-M, C-H
e4	Chi^2 =6.43	2	0.04	C-M, C-H
r2	Chi^2 =11.25	2	0.00	C-H

Table 12: Effects of time on glucosidase activity tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). The table below shows the pairwise comparison between particular weeks of the experiment (c = colonization; e1 – e4 = nutrient enrichment; r2 = recovery phase). Significant values are highlighted.

Epilithic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 32.4$	5	0.00
Moderate	$Chi^2 = 51.60$	5	0.00
High	$Chi^2 = 61.20$	5	0.00

Epipsammic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 32.40$	5	0.00
Moderate	$Chi^2 = 41.35$	5	0.00
High	$Chi^2 = 22.12$	5	0,00

Epilithic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	**0.02**	1.00	1.00	**0.02**	**0.02**
Moderate	1.00	1.00	1.00	**0.00**	**0.02**
High	**0.02**	**0.02**	**0.02**	**0.00**	**0.00**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.02**	**0.02**	**0.00**	1.00	**0.02**	**0.02**	**0.02**
Moderate	**0.02**	1.00	**0.00**	1.00	**0.00**	**0.00**	**0.00**
High	**0.02**	**0.02**	**0.00**	1.00	**0.02**	**0.02**	1.00

Epipsammic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	1.00	**0.02**	**0.02**	1.00	1.00
Moderate	1.00	**0.02**	**0.02**	**0.02**	1.00
High	1.00	1.00	**0.02**	**0.02**	**0.02**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.02**	**0.02**	1.00	**0.02**	1.00	**0.02**	1.00
Moderate	0.06	1.00	1.00	1.00	**0.00**	**0.00**	1.00
High	0.06	0.06	1.00	**0.02**	**0.02**	**0.02**	

4.6 Xylosidase

4.6.1 Epipsammic biofilm

Xylosidase could just be detected in epipsammic biofilm (Figure 15). Temporal development revealed a continuous increase of xylosidase activity in all groups throughout enrichment phase, which implies, that colonization was not completed yet (Table 14). However, measurements clearly showed effects of nutrient addition as the most prominent increase between the certain weeks was detected in nutrient enriched flumes. The recovery phase was characterized by a further increase. Either by comparing the particular groups during enrichment and recovery phase or by comparing the particular phases within a group, all these arrangements significantly differed amongst each other (*Table 13, Table 14*).

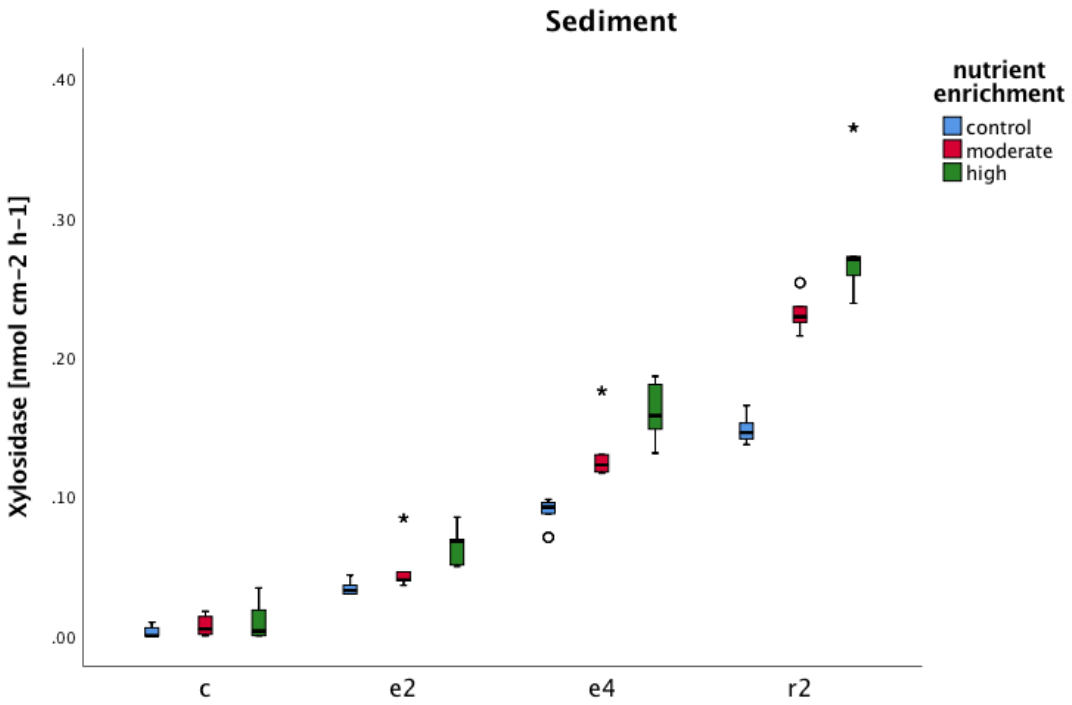

Figure 15: Xylosidase activity in epipsammic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

Table 13: Effects of nutrient enrichment on xylosidase activity in epilithic and epipsammic biofilm tested by median test. Significant differences are based on a Chi^2 test ($p < 0.05$). Pairwise comparison was made between control group (C), moderate (M) and high (H) enrichment.

Epipsammic

	Test statistic	**df**	**Asymptotic sig.**	**Pairwise Comparison**
c	$Chi^2 = 1.61$	2	0.45	-
e2	$Chi^2 = 30.54$	2	0.00	C-M, C-H, C-M
e4	$Chi^2 = 30.54$	2	0.00	C-M, C-H, C-M
r2	$Chi^2 = 30.54$	2	0.00	C-M, C-H, C-M

Table 14: Effects of time on xylosidase activity tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). The table below shows the pairwise comparison between particular weeks of the experiment (c = colonization; e1 – e4 = nutrient enrichment; r2 = recovery phase). Significant values are highlighted.

Epipsammic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 90.00$	5	0.00
Moderate	$Chi^2 = 90.00$	5	0.00
High	$Chi^2 = 90.00$	5	0.00

Epipsammic

	c – e2	**c – e4**	**c – r2**
Control	**0.00**	**0.00**	**0.00**
Moderate	**0.00**	**0.00**	**0.00**
High	**0.00**	**0.00**	**0.00**

	e1 – e2	**e1 – e4**	**e2 – e4**	**e4 – r2**
Control	**0.00**	**0.00**	**0.00**	**0.00**
Moderate	**0.00**	**0.00**	**0.00**	**0.00**
High	**0.00**	**0.00**	**0.00**	**0.00**

4.7 Leucine

4.7.1 Epilithic

The release of leucine showed a clear positive reaction to nutrient enrichment in epilithic biofilm which could already be observed in e1, where concentrations in treated groups were significantly higher than in control group (Figure 16). Although leucine activity in epilithic biofilm continuously increased independently of the treatment, conspicuous differences due to nutrient enrichment could first be evidenced in week e3 in high-enriched flumes. The temporal development towards the end of enrichment phase showed a further steep increase of nutrient treated groups but also a slight increase of control group. Nevertheless, all groups significantly differed amongst each other throughout the whole enrichment phase with highest leucine activity detected in high-enriched flumes followed by moderate enriched flumes and control group. During recovery phase, enzyme activity of treated flumes considerably decreased and led to an approximation of moderate enriched flumes with control group. However, measured activities in r2 still exceeded leucine activities during colonization phase.

4.7.2 Epipsammic

Epipsammic biofilm (Figure 17) also showed clear effects of nutrient enrichment but in contrary to epilithic biofilm, where released leucine continuously increased throughout the whole enrichment phase, epipsammic biofilm samples first showed an increase within the first two weeks of enrichment followed by a decrease towards the end of enrichment phase. However, highest release of leucine was always measured in high-enriched flumes as it could also be observed in epilithic biofilm. By comparing both sediment types, leucine activity in epilithic biofilm was considerably higher and differences between all groups were more striking. During recovery phase, high enriched flumes in epipsammic biofilm further increased the release of leucine and significantly differed to moderate enrichment as well as control group.

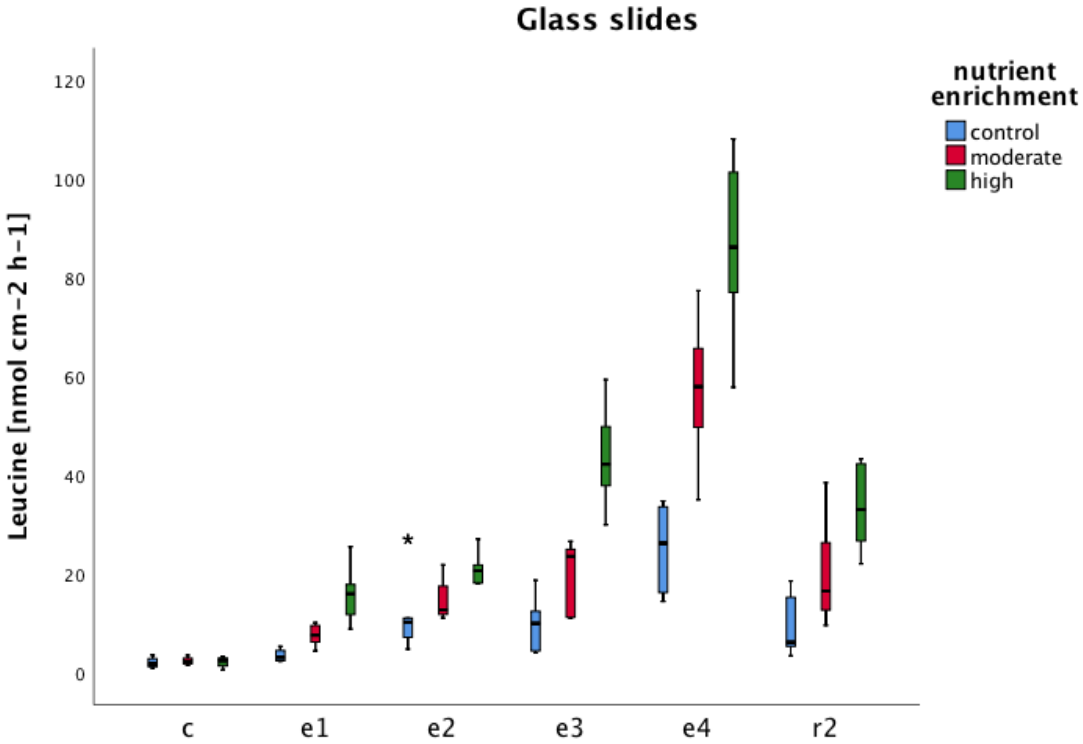

Figure 16: Leucine activity in epilithic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

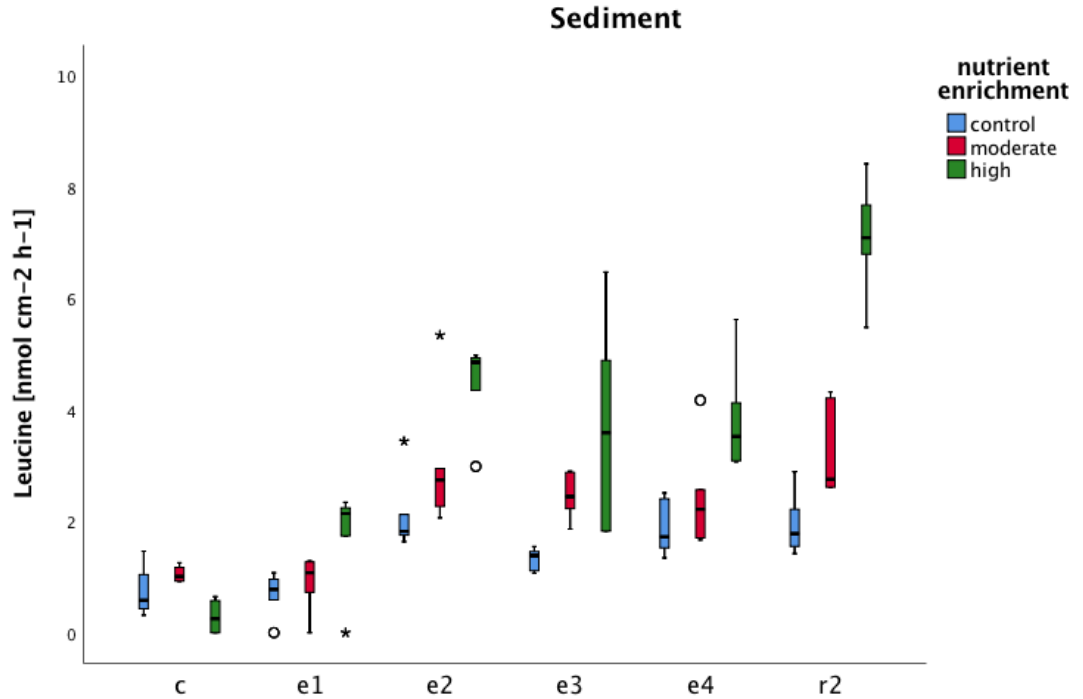

Figure 17: Leucine activity in epipsammic biofilm showing control group, moderate and high nutrient enrichment throughout the whole experiment. Shown are median, 10, 25, 75 and 90 % percentiles and outlier (n = 45 per treatment and date).

Table 15: Effects of nutrient enrichment on leucine activity in epilithic and epipsammic biofilm tested by median test. Significant differences are based on a Chi^2 test ($p < 0.05$). Pairwise comparison was made between control group (C), moderate (M) and high (H) enrichment.

Epilithic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 6.000$	2	0.05	C-H
e1	$Chi^2 = 27.60$	2	0.00	C-M, C-H, M-H
e2	$Chi^2 = 25.71$	2	0.00	C-M, C-H, M-H
e3	$Chi^2 = 30.54$	2	0.00	C-H, M-H
e4	$Chi^2 = 20.89$	2	0.00	C-M, C-H, M-H
r2	$Chi^2 = 30.54$	2	0.00	C-H, M-H

Epipsammic

	Test statistic	df	Asymptotic sig.	Pairwise Comparison
c	$Chi^2 = 6.43$	2	0.82	-
e1	$Chi^2 = 6.41$	2	0.04	M-H
e2	$Chi^2 = 11.25$	2	0.00	C-H, M-H
e3	$Chi^2 = 11.25$	2	0.00	C-M, C-H
e4	$Chi^2 = 6.43$	2	0.04	C-H
r2	$Chi^2 = 11.25$	2	0.00	C-H

Table 16: Effects of time on leucine activity tested by using a median test. Significant differences are based on a Chi^2 test ($p < 0.05$). The table below shows the pairwise comparison between particular weeks of the experiment (C = colonization; e1 – e4 = nutrient enrichment; r2 = recovery phase). Significant values are highlighted.

Epilithic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 45.6$	5	0.00
Moderate	$Chi^2 = 43.75$	5	0.00
High	$Chi^2 = 62.97$	5	0.00

Epipsammic

	Test statistic	df	Asymptotic sig.
Control	$Chi^2 = 58.17$	5	0.00
Moderate	$Chi^2 = 48.55$	5	0.00
High	$Chi^2 = 48.55$	5	0.00

Epilithic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	0.22	**0.00**	**0.00**	**0.00**	**0.00**
Moderate	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
High	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.00**	1.00	**0.00**	1.00	**0.02**	**0.02**	**0.02**
Moderate	**0.00**	**0.00**	**0.00**	1.00	**0.00**	**0.00**	**0.02**
High	**0.02**	**0.00**	**0.00**	**0.00**	**0.00**	**0.02**	**0.00**

Epipsammic

	c – e1	c – e2	c- e3	c – e4	c – r2
Control	1.00	**0.00**	1.00	**0.00**	**0.00**
Moderate	1.00	**0.00**	**0.00**	**0.00**	**0.00**
High	**0.00**	**0.00**	**0.02**	**0.00**	**0.00**

	e1 – e2	e1 – e3	e1 – e4	e2- e3	e2 – e4	e3 – e4	e4 – r2
Control	**0.00**	**0.02**	**0.00**	**0.00**	1.00	**0.02**	1.00
Moderate	**0.00**	**0.00**	**0.00**	1.00	1.00	1.00	**0.02**
High	**0.00**	1.00	**0.00**	1.00	**0.02**	1.00	**0.02**

4.8 Short term nutrient addition

Although all flumes received the same amount of phosphate (20 ml of a 100 mg/l P-PO4 solution), the first measurement of SRP concentrations after 1 hour (starting point after full mixing) already showed significant differences between the control and the treatment flumes (Figure 18). Mean concentrations in the control flumes were about 60 µg L-1, while both treatment groups showed mean concentrations of approximately 20 µg L-1. Thus, the control group had the lowest phosphate uptake. Two hours after injection there was nearly no phosphate left, regardless of the group (Figure 18).

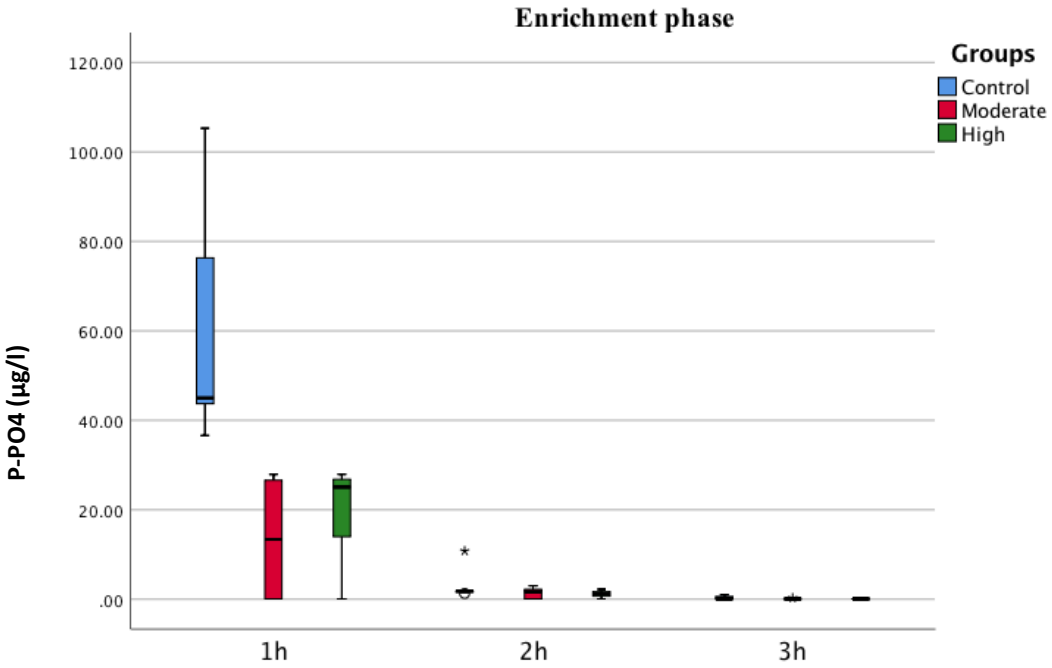

Figure 18: This graph shows the remaining phosphate concentrations after short term nutrient injection in the particular groups at the end of enrichment phase. Shown are median, 10, 25, 75 and 90 % percentiles and outliers (n per group = 5).

For testing nutrient uptake within the recovery phase (Figure 19), we added 40 instead of 20 ml of a 100 mg/l P-PO4 solution to all flumes as we expected a fast uptake. This graph shows, that uptake was still lowest in the control group and highest in the former high nutrient enriched group. Nevertheless, after the 3rd hour, almost the entire phosphate had been taken up within all groups.

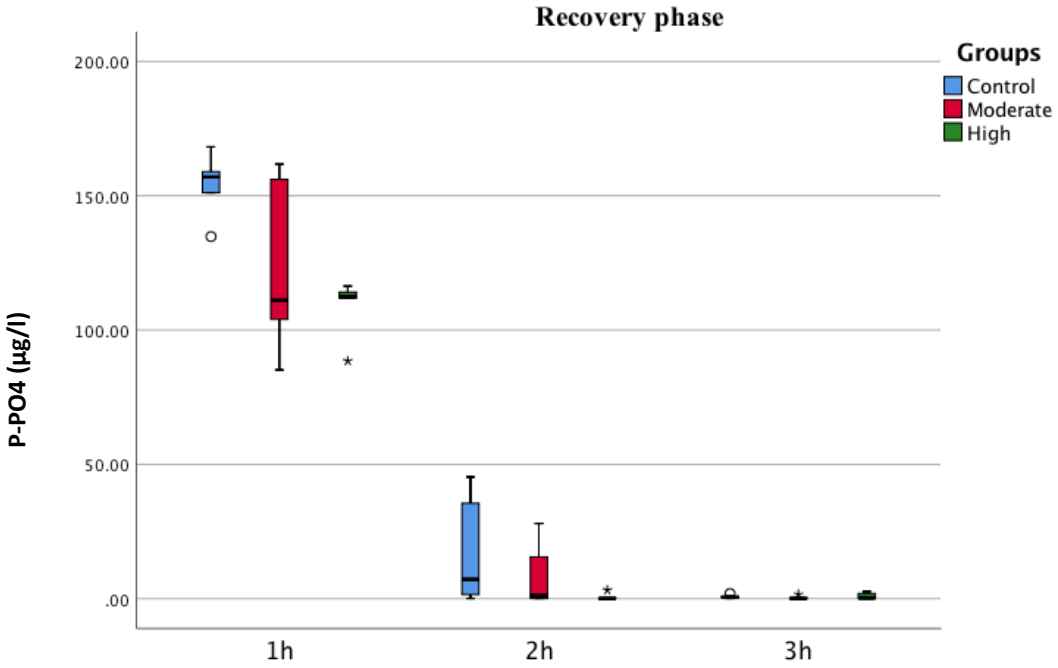

Figure 19: This graph shows the remaining phosphate concentrations after short term nutrient injection of particular groups at the end of recovery phase. Shown are median, 10, 25, 75 and 90 % percentiles and outliers (n per group = 5).

5. Discussion

5.1 Effects of nutrient enrichment on biomass development of epilithic and epipsammic biofilms

One of the main hypothesis of this thesis predicted that algal and bacterial biomass will increase with moderate and high long-term nutrient enrichment of acetate, phosphate and nitrate under controlled light conditions in the laboratory. Applied statistical analysis have proven a positive effect of continuous moderate and high nutrient enrichment on algal biomass in both substrate types. However, nutrient enrichment seemed to have larger effects on epilithic biofilms than on epipsammic biofilms, which could be seen by the higher biomass production, but also by the faster and stronger response of the particular treatments within epilithic compared to epipsammic biofilm (Table 6). As expected, chlorophyll-a in epilithic biofilm of high or moderate nutrient enrichment continuously increased until the end of the experiment without signs of a flattening of the curves indicating a saturation. In contrast, algal growth in epipsammic biofilms was generally lower and seemed to stagnate up from the second week of enrichment. A similar pattern of results was also found by Sabater et al., (2011) and Romaní et al., (2004) who explained such differences between substratum types by the higher turnover of the Epipsammic substrate but also by the fact, that detritus accumulation might have negative influences on algal growth. However, these studies were carried out in streams with natural conditions and findings may not be as relevant for our experimental setup. Anyway, an explanation for the higher epilithic biomass production might be the stable surface of the slides which supports a denser biofilm development. Despite the low flow velocity, fine sediment was always in a little motion and thus less appropriate for biomass production.

Independently of the substrate type it could be seen, that referring to the effects of different nutrient levels, moderate and high nutrient enrichment lead to a faster increase of chlorophyll-a after the first week of enrichment. The increased nutrient levels mainly affect autotrophs and their structural and functional characteristics, e.g. a shift in species composition to taxa which are more tolerant and which have higher nutrient uptake rates (Felisberto et al., 2012; Whitton and Kelly, 1995). Nevertheless, the additional phosphate injection in week e3 and e4 may indicate, that algal biomass was possibly limited by phosphate as there is a steep increase noticeable from e2 to e3 whereas the development from e3 to e4 may show the beginning of a saturation.

Bacterial biomass production was also expected to increase as former studies showed that inorganic nutrients, which positively affect algal growth, are indirectly linked with bacterial biomass production via DOC uptake deriving from algae (Bell, 1983; Rier and Stevenson, 2002). However, we also know, that there is a direct effect of inorganic nutrients on heterotrophic bacteria as several studies have proven their high affinity to inorganic N and P (Kirchman, 1994; Sabater et al., 2011, Currie and Kalff's, 1984b). Referring to our results, a mutualistic relationship between algae and bacteria seems to exist in *epilithic* biofilm. As we assumed, bacterial biomass simultaneously increased with algal growth within the particular treatments and also showed a very similar development during recovery phase. In contrast to epilithic biofilms, bacterial growth in *epipsammic* biofilms – which was by far lower than in epilithic biofilms - decreased after the colonization phase and rarely increased during the enrichment phase. Possible explanations might be a higher instability of the substratum, but also carbon limitation, which could already be observed by Sabater et al., (2011) and Romaní et al., (2004). As bacteria normally react rather fast to nutrient enrichment there might have been some additional factors like e.g. *too short colonization phase as pre-cleaned substrate was used, the quantity of substrate used within a flume but also temperature, oxygen or light conditions* which finally influenced these results.

Overall, the hypothesis that algal and bacterial biomass will increase with moderate and high nutrient enrichment can generally be accepted but strongly depend on the substrate type.

5.2 Effects of nutrient enrichment on enzyme release

5.2.1 Extracellular enzymes

Extracellular enzyme activity was expected to vary concerning the substrate type but also referring to the particular nutrient enrichment. Former researches already showed that enzymes like e.g. peptidase and beta-glucosidase, which are involved in the breakdown of algal biomass, are very characteristic for epilithic biofilms as this substrate type is rather suitable for high algal growth (Ainsworth and Goulder, 2000). This could be proven by Anna M. Romaní et al. (2004) who observed the coupled increase of peptidase, algal as well as bacterial biomass and interpreted this observation as an elevated need of heterotrophic bacteria on proteinaceous compounds. Enzymes like e.g. peroxidase, xylosidase and cellobiohydrolase mainly occur in the epipsammon where they play an important role in the microbial decomposition of complex polysaccharides (Allison and Vitousek, 2005; Gulis et al., 2004; Romaní et al., 2004; Sabater et al., 2011). All these enzymes

are known to be primarily released by bacteria in situations where nutrients are abundant (Allison and Vitousek, 2005). However, to our knowledge, the effects of moderate and high long-term enrichments of acetate, phosphate and nitrate on the activity of extracellular enzymes has not been studied systematically so far (Gulis et al., 2004; Stanley et al., 2012). This raised the question whether the release of extracellular enzymes will continuously increase with increasing supply of inorganic nutrients and acetate also referring to various substrate types or whether we may observe saturation at very high nutrient and acetate levels. As expected, the epilithon – which was characterized by a stable and high algal biomass production throughout the enrichment phase – largely supported former findings (Anna M. Romaní et al., 2004) as the release of beta-glucosidase as well as leucine-aminopeptidase increased with increasing nutrient and DOC supply and, consequently, with the availability of algal biomass. Especially peptidase reacted very fast to the nutrient enrichment which might be explained by the fast uptake of peptide molecules coming from algal material (Francoeur and Wetzel, 2003). Within the recovery phase, the released glucosidase and peptidase also seemed to follow the lower availability of algal biomass as the release slightly decreased. Regarding the epipsammon, leucine-aminopeptidase again clearly increased during the enrichment phase concurrently with the higher algal biomass and thus the higher availability of labile organic material. In contrast, ß-glucosidase was characterized by a general decrease in all treatments between the first and the third week of the enrichment phase. A possible explanation therefore could be the lower bacterial biomass within this time period and the fact, that the additional DOC enrichment may have covered their carbon demand (Lutz et al., 2012). In the last week of enrichment phase, bacterial biomass slightly increased and also positively affected the release of glucosidase. However, the fact, that glucosidase also decreased in the control group might be an evidence of additional factors influencing the release of ß-glucosidase, such as temperature. The interaction of temperature and beta-glucosidase was proven by Fenoy et al. (2016), who found out, that the release of beta glucosidase double by increasing temperature by 10 degrees. Anyway, by disregarding this overall decrease in week 2 and 4, the treated flumes showed higher activities of glucosidase than the control group at the end of the experiment (week 4) and during the recovery phase. The delayed response of glucosidase to the enrichment may be due to the higher organic matter accumulation derived from the epilithic biofilms (Sabater et al., 2011).

Xylosidase – which is involved in the degradation of hemicellulose (Gulis et al., 2004) - was solely detected in epipsammic biofilms which can be explained by the fact, that wooden sticks were buried in the sediment providing an additional food source rather for the epipsammic than the epilithic biofilms. Although all treatments showed an increase in xylosidase throughout the whole experiment, the treated flumes had a significantly higher release of xylosidase than the control which implies an increased decomposition within the enriched flumes (Greenwood et al. 2007; Gulis et al., 2004). Overall, tested hypothesis can clearly be answered as all extracellular enzymes, which are involved in the degradation of organic matter, showed an increased release with moderate and high nutrient enrichment whereas high nutrient enriched flumes had a significantly higher release of xylosidase than moderate enriched flumes. Despite the fact, that flumes were continuously enriched with inorganic nutrients and organic carbon, these results would also indicate that the DOC uptake was not saturated and thus decomposition was stimulated by the enrichments.

A main hypothesis stated that phosphatase – which is primarily released by algae in situations with low phosphorus availability – will decrease with phosphorus loading (Allison and Vitousek, 2005; Battin et al., 2009; C. E. Davis and Mahaffey, 2017; Lindahl and Hospital, 1984; Rier et al., 2014; Romaní et al., 2004; Sinsabaugh and Moorhead, 1994). Results of this study showed a strong probability that this hypothesis can be supported. Released phosphatase between week 3 and 4 of nutrient enrichment showed a slight increase within moderately enriched flumes which could imply a limitation of phosphate. On the other hand, phosphatase in highly enriched flumes slightly decreased which could represent a saturation.

Alkaline phosphatase activity in epipsammic biofilms also showed signs of P saturation in the highly enriched group and a possible P limitation in the moderately enriched group, albeit the differences were not significant. With the additional phosphate injection in week 3, which was executed due to the fast P uptake, APA radically decreased in epipsammic biofilms, but not in epilithic biofilms. The different response in epipsammic and epilithic biofilms to the increased P supply raise the question of the cause for this decerase. A possible explanation might be the considerably lower algal biomass in epipsammic biofilm which also implies a lower demand of phosphorous. Thus, while the increased P supply was sufficient for the thinner epipsammic biofilms, epilithic biofilmshad a P demand still exceeding the supply. (Riber and Wetzel, 1987) Focussing on the phosphatase development during the recovery phase it can be seen, that at least

APA in epilithic biofilm showed a very similar trend as the algal biomass. Overall it can be assumed, that these results show a positive trend and partly support the stated hypothesis. However, to get more detailed results to answer the question, if a permanent phosphorous enrichment will decrease phosphatase it would have been necessary to add higher concentrations of phosphorous as a saturation was not fully reached within all flumes.

5.2.2 Nutrient uptake

The last hypothesis of this study predicted, that nutrient uptake will increase with moderate and high nutrient enrichment. This assumption could also be confirmed by a nutrient uptake experiment during the enrichment phase which demonstrated, that the measured phosphate concentrations one hour after the injection were clearly lower in the moderate and highly enriched flumes than in the control group, indicating a higher short term nutrient uptake within the treatment flumes. Previous studies already emphasized the importance of algal biomass as an important biotic factor influencing nutrient uptake (Angelo and Webster, 1991). As phosphate was the only nutrient used for this short term nutrient experiment it can be assumed that adsorption as an abiotic factor may also have influenced nutrient uptake in the flumes (Niyogi et al., 2004) . Investigations have shown that phosphorous strongly adsorbs to sediment with a smaller grain size. In such aquatic systems, abiotic P-uptake has even more effect than biotic P-uptake (Lottig and Stanley, 2007). During the recovery phase, the P uptake experiment showed no significant differences between the different flumes anymore which can be explained by the decrease in algal biomass and thus the lower demand of nutrients.

5.2.3 Functioning of agricultural streams

Our experiment represents the impacts of a long-term nutrient and organic carbon enrichment under non light-limiting conditions. Contrary to expectations, neither moderate nor high enriched group showed any signs of a saturation concerning nutrient uptake except the decrease of phosphatase, which could indicate a saturation of phosphate. We could observe, that long term enrichment lead to an adaption of the communities through an increasing biomass with a consequently higher nutrient demand, which could be supported by the fast nutrient uptake. Previous studies interpreted these observations by an altered community composition and a higher biological activity (Bernot and Dodds, 2005; García et al., 2016; O'Brien et al., 2007), which raises nutrient uptake and thus shows a certain resistance to high nutrient loading (Niyogi et al., 2004). Nevertheless, results

strongly differed amongst epilithic and epipsammic biofilms due to their different structural and functional characteristics. Whereas epipsammic biofilms are known to have a less complex structure, epilithic biofilms show a more complex structure and a tight spatial interaction (Romaní and Sabater, 2001; Sabater et al., 2006). Another important information could be deduced from results during recovery phase where both biofilms showed a slow decrease after stopping nutrient enrichment which may indicate, that there are still nutrients available through accumulation or adsorption (Stephen Carpenter et al., 1998; Withers and Haygarth, 2007). Overall, this experimental framework provided a good possibility to investigate effects of long-term nutrient enrichment and easy available carbon without light limiting effects and other disrupting factors like macrozoobenthos which feed on algae. Focusing on more natural conditions, future research needs to address light limitation as a strong influencing factor but also DOC limitation, as it can occur in a more complex form (Baker et al., 1999)

5.2.4 Improvements for experiments

Although this experiment provides a good overview of the impacts of nutrient loading to in-stream nutrient uptake and metabolic processes it could be seen, that e.g. epipsammic biofilm colonization and activity partly showed unexpected trends. This might be explained by the experimental set up and may require some modifications for further experiments as e.g. a prolonged colonization phase to guarantee a certain maturity of the biofilms (Peterson et al., 1985) or more stable temperature conditions (Table 2) (Fenoy et al., 2016). Furthermore, the intervals of added nutrients should probably be reconsidered as e.g. phosphate as the limiting factor was taken up very fast, which made it difficult to keep elevated levels and assess long term enrichment efects. Further experiments could reveal at what time and which concentration levels a saturation can be observed for both substrate types. For testing short term nutrient uptake it would also be useful to use nitrate instead of phosphate as phosphate is the limiting factor and thus taken up very fast (Jansson, 1988).

6. Conclusion

Overall this study presented an adaption of algae and bacteria to moderate and high nutrient loading for four weeks which is very important for the self-purification of a stream (Besemer et al., 2013). As we assumed, algal and bacterial biomass was highest in high enriched flumes, followed by moderate enriched flumes, but saturation effects could not be reached. Furthermore, the increase in biomass enhanced nutrient uptake (Niyogi et al., 2004) and thus ensured a certain resistance to pollution. Strong differences were visible between the growth of autotrophs and heterotrophs but also referring to the different substrate types, as epilithic biofilms showed a higher response than epipsammic biofilms. The data also suggest, that phosphatase showed signs of a saturation above a certain threshold of µg P/l and supports the assumption, that decomposition is stimulated by the enrichment of labile DOC.

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Eidesstattliche Erklärung

Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

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