Effects of multiple stressors on the structure and function of stream benthic communities

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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To Jeanne DECOSTAZ

Declaration

I, **Noël Juvigny-Khenafou**, wish to declare that this thesis is my own independent work. It is being submitted in partial fulfilment of the requirement for the award of the degree of PhD. I further declare that this work has never been submitted for any degree at this or any other University, and that the thesis is presented with the consent of my supervisor. Works by other authors, which served as information source, have been duly acknowledged by references to the authors. The views expressed are my own. I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organizations.

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Noël Juvigny-Khenafou

1st March 2020

Abstract

The development of human activities has intensified and diversified the pressures applied to freshwater ecosystems. Particularly, land use stressors have been very pervasive and widespread. As a result, most freshwater systems are now under the influence of anthropogenic stressors. For instance, agricultural development and urbanisation have elevated the nutrients levels, facilitated the accumulation of chemicals, modified the natural flow velocities and promoted runoffs and sediment loads. Further, stressors often interact with each other, complicating the prediction of their effects on communities and ecosystem functioning; flow velocity and discharge reduction facilitate the accumulation of chemical and fine sediments. In order to evaluate the effect of multiple stressors and inform decision makers, investigations have been conducted worldwide on different trophic levels and ecosystem processes. Most notably, microbes, algae and macroinvertebrates have often been studied in isolation using taxonomic and now molecular methods. However, communities are made of complex population dynamics involving all trophic levels over time, and emergent ecosystem properties such as decomposition or net productivity are the result of multiple interactions between biotic and abiotic parameters. This calls for more holistic approaches encompassing as many facets of biodiversity as possible.

To investigate the effect of multiple land use stressors associated with agriculture and urbanisation, a highly replicated streamside field mesocosm experiment was built and performed in a near-pristine montane environment. The work was conducted in Autumn 2018 in the Jiulongfeng Nature Reserve, Huangshan, Anhui (China) and consisted of 64 experimental units naturally colonised by stream organisms for 3 weeks. I used a 4-factor full-factorial design, manipulating fine sediment deposition, flow velocity and nutrient concentration at two sampling times (2 and 3 weeks of exposure). Linear models were then applied to analyse the temporal response of microbial communities associated with both leaf litter decay and benthic biofilm formation, as well as the benthic macroinvertebrate communities. Additionally, to infer the emergent properties and functional characteristics of the different communities, four commonly used functional indices were investigated: (i) leaf litter decomposition in Chapter 2, (ii) databased predicted functional profile in Chapter 3, (iii) functional traits and (iv) functional diversity in Chapter 4. I then expanded my reflection from

the knowledge acquired in the experimental side of my programme and outlined a novel framework to tackle multiple stressors interactions in riverine networks (Chapter 5).

The molecular analysis of microbial communities showed different impacts on species composition of the different stressors between microbes associated with leaf-litter decomposition and with biofilm development. Indeed, whilst nutrient enrichment and flow velocity reduction appeared to be the most pervasive factors affecting microbial decomposers communities on leaf substrates, fine sediment deposition and flow velocity reduction were most important for biofilm communities. Fine sediment deposition and flow velocity reduction were also the dominant factors driving macroinvertebrate community composition. Furthermore, both molecular analyses indicated that microbial clusters could be identified in response to the dominant stressors. In terms of interactions, 2-way interactions involving sediment and flow velocity reduction (sediment × flow velocity reduction) or nutrient enrichment and sediment (nutrient enrichment × sediment) were the most pervasive overall; 3-way interactions involving nutrient enrichment, sediment deposition and flow velocity reduction (nutrient enrichment × sediment × flow velocity reduction) were also detected. Furthermore, temporal dynamics were also fairly widespread, highlighting the importance of integrating a temporal factor in multiple stressor studies. Finally, in accordance with the existing literature, changes in abiotic factors often led to functional rearrangements of the different communities underlying the environmental filtering and niche selection processes operating in the system.

From integrating the findings of this thesis into the wider subject area, I suggest ecosystem approach to multiple stressor interaction research. Specifically, I propose that future work adopt a spatiotemporal framework better integrating the energy fluxes across trophic levels and the flow of resources and material through riverine networks. Further, combining alpha diversity indices with functional traits aids understanding of the mechanisms that yield emergent ecosystem properties, such as productivity. Together, it is anticipated that spatiotemporal networks and functional measurements will facilitate prediction of the future stability of freshwater systems under stressor accumulation.

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List of Figures

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This thesis is based on the following publications:

I. Zhang, Y., Juvigny-Khenafou, N.P.D., Xiang, H., Lin, Q., Wu, Z. 2019. Multiple Stressors in China's Freshwater Ecoregions. in: Sabater, S., Elosegi, A., Ludwig, R. (Eds.), Multiple Stressors in River Ecosystems. Elsevier, pp. 193–204.

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Author's contribution

Paper I:

YZ conceived the idea. YZ, NJK, HX, QL lead the writing with contributions from ZW.

Paper II, III, IV:

NJK and JP conceived the studies with contributions from YZ. NJK collected data and ran the experiments with contributions from SM in paper IV. NJK and CM analysed and interpreted the data. NJK and CM lead the writing with contributions from JP, DA, YZ, NW and SVB (paper II), SM (paper III).

Paper V:

NJK and EH conceived the idea and lead the writing with contribution from JP, DA, NW and YZ.

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1. Chapter 1: General Introduction

1.1. Stressors in freshwater systems

Access to freshwater resources has always influenced human distribution and density across landscapes. As such, land use modifications have historically been dominant sources of stress for freshwater habitats (Dudgeon et al., 2006; Hering et al., 2015; Nõges et al., 2016; Reid et al., 2019). Indeed, human activities exploit freshwater resources - and their adjacent terrestrial environments - for food production (agriculture and fisheries), drinking water, energy and transport amongst others. In ecology, 'stressors' can be defined as any anthropogenic activity resulting in environmental change that will take the studied system outside of its normal operational range (Sabater et al., 2019). With the constant growth in human global population and activities, there is an increase in the diversity and the intensity of stressors applied to freshwater environments (Reid et al., 2019). Consequently, freshwater ecosystems are some of the most threatened ecosystems in the world with a rate of biodiversity loss higher than any other system (McRae et al., 2017). This is of particular concern considering that freshwater ecosystems harbour 6% of all described species despite only representing 0.01% of the World's waters and 0.8% of the Earth surface area (Dudgeon et al., 2006).

Freshwater biodiversity is modulated by physicochemical parameters that shape communities and regulate processes over time and space (Harvey and Altermatt, 2019; MacDougall et al., 2018; Widder et al., 2014). A modification of these environmental parameters leads to abundance and diversity changes (Mustonen et al., 2016; Piggott et al., 2015c; Salis et al., 2017). In return, ecological processes and functioning are being modified and new states are being reached (Gordon et al., 2008; Manning et al., 2018; Piggott et al., 2015a). Most notably, and central to this PhD thesis, stressors associated with urbanisation and agricultural intensification have elevated the inputs of dissolved nutrients, promoted the deposition of fine sediment and altered stream discharge and current velocity due to water diversion and channel modifications (Elbrecht et al., 2016; Matthaei et al., 2010; Wagenhoff et al., 2012). In this intricate network of biological, physical and chemical interactions, stressors upset the finely tuned balance in stream and river dynamics and generate complex responses sometimes referred to as 'ecological surprises' (Lindenmayer et al., 2010). The level of complexity

depends on the system being studied and is a function of the mode of action of the different stressors involved, which is likely to be exacerbated by the effects of global changes such as climate change (Mantyka-Pringle et al., 2019; Piggott et al., 2015c; Tiegs et al., 2019).

In China, the rapid economic, urban and agricultural intensification developments have put a lot of pressure on freshwater systems. As a result, most rivers and groundwater sources are polluted (Han et al., 2016; Liu and Diamond, 2005). For instance the transition from small traditional farmlands to large-scale industrialised farms relying on synthetic fertilisers has increased nutrient inputs to rivers by a factor 2 to 45 for country wide sub-basins between 1970 and 2000 (Strokal et al., 2016). Water abstraction is also a dominant stressor in Chinese streams and rivers with large dam and translocation projects being built across the country to supply energy and to bring water to regions with a shortage (Dudgeon, 2011; Wilson et al., 2017). There are more than 50,000 dams of a variety of sizes in the Yangtze River basin alone (Li et al., 2013) and China's South–North Water Transfer Project (SNWTP) has the potential to transfer as much as $4.48e_{10}$ $m_3.yr_{-1}$ of water from the Yangtze River basin to the Yellow River basin (Wilson et al., 2017). Finally, sediment deposition has been facilitated by the alteration of river flow regimes but also soil erosion as a result of land use modification and climatic events (Li et al., 2013; Zuo et al., 2016). For instance, approximately 162 $Mt.yr^{-1}$ of sediment were trapped by the Three Gorges Dam between 2003 and 2007 (Hu et al., 2009).

Despite these pressing environmental issues, little research in China has sought to investigate the effects of multiple stressors on freshwater communities in comparison to the size of the country. For instance, an advance Web of Science search (Accessed 15/01/2020) using the function TS=(stress AND China) AND TS=(stream OR lake OR river OR freshwater), revealed that four of the largest and most research active countries in the European Union (Spain, Germany, Sweden and France) have published the equivalent of 68.8% of the total China multiple stressor research output despite representing only 19.3% of China's total area. Anthropogenic pressures associated with urbanisation and agriculture are particularly preponderant in eastern lands which are the most densely populated and concentrate the industrial and agricultural activity of the country (Zhang et al., 2019). Although concern for the management of freshwater resources is high (Han et al., 2016; Speed et al., 2016), stressor research in China suffers from having a patchy distribution of monitoring, and limited investigations of the interactive effects between stressors. Therefore, there is little

understanding of the synergistic or antagonist nature of the interactions, nor of their magnitude. Due to the complexity and unpredictability of the interactions between stressors, this represent a clear knowledge gap which may hinder the long-term success of managerial decisions (Côté et al., 2016; Crain et al., 2008; Nõges et al., 2016).

1.2. The complexity of interactions between stressors

The origins of a biological response to changing conditions can be broad and multiple. As an example, eutrophication, which is characterised by an excessive plant and algal growth, can be triggered by changes in one or more limiting growth factors needed for photosynthesis (Chislock et al., 2013; Schindler, 2006); these can be sunlight, carbon dioxide, and nutrient fertilizers for instance. The effects that individual stressors have, and the responses of ecological processes and communities, are well studied. Taking the focal stressors of this thesis as examples, fine sediment deposition and suspension (< 2 mm, Rabení et al., 2005) reduces habitat heterogeneity by infilling the interstitial spaces in the benthos, reduces the O_2 availability, reduces light penetration, and smothers the gills of fish, invertebrates, algal filament stalks or biofilm formations (Piggott et al., 2015c; Wood and Armitage, 1997). This is particularly detrimental to organisms with exposed fragile breathing structures or high O_2 requirements (Wood and Armitage, 1997). Sediment deposition also changes the physical characteristics of the substratum on which biofilm can attach (Battin et al., 2016). Both sedimentation and reduced flow velocity limit the exchange of resources between the water column and benthic habitats by creating a physical barrier and increasing boundary layer thickness (Barker Jømgensen and Des Marais, 1990; Stevens and Kurd, 1997), whereas eutrophication favours the development of microbes associated with algal blooms (Piggott et al., 2015b). Eutrophication also produces subsidy-stress response gradients in invertebrate and microbial communities and activity (Güsewell and Gessner, 2009; Wagenhoff et al., 2012; Woodward et al., 2012). Finally, water scarcity modifies the physical habitat and the diffusion of material and resources through flow-velocity reduction (Calapez et al., 2018; Harvey et al., 2017a; Wu et al., 2019).

The challenge comes when one tries to predict the cumulative effect of stressors. The first step in studies of multiple stressors requires the development of a null model that best describes the mechanisms by which the stressors are thought to operate, i.e. the linear combination of the stressor individual effects in the absence of interaction (Schäfer and Piggott, 2018). The effect

of stressor interactions is then assessed by comparing discrepancies from the null model. For statistical ease, the most commonly applied null model has been the additive model (when the response to stressors add to one another). Most reported cumulative effects deviate from the additive model and are called non-additive (Crain et al., 2008, Nõges et al., 2016). They are described as antagonistic when the cumulative impact is smaller than the expected product of the response of each stressor alone, and synergistic when it is higher (Crain et al., 2008; Folt et al., 1999). This basic description of the additive model interaction effects was later re-visited by Piggott et al. (2015d), highlighting the problems associated with individual stressors acting in opposite directions. In this particular, but not unusual, situation the definition used of synergism and antagonism becomes paradoxical as what is synergistic to one stressor is antagonistic to the other and *vice-versa*. As such the new concepts of positive and negative antagonisms/synergisms were introduced and summarised below:

- If an interaction of stressors is less positive than expected from the additive model, then it is *positive antagonistic*;
- If an interaction of stressors is less negative than expected from the additive model, then it is *negative antagonistic*;
- If an interaction of opposing stressors is more positive than expected from the additive model, then it is *positive synergistic*;
- If an interaction of stressors is more negative than expected from the additive model, then it is *positive antagonistic*.

The mechanisms involved between the interaction of stressors and the observed biological responses are particularly hard to unfold. Indeed, species may respond in various ways to different sets of stressors due to differences in evolutionarily or ecologically derived tolerances (Vinebrooke et al., 2004). The impact of stressors also can change in the presence of other stressors. For instance, nutrient enrichment effects on decomposition and spore production in fungal aquatic decomposers change along temperature gradients (Fernandes et al., 2014). Finally, whole community responses differ as a result of the changing interactions between species populations (Crain, 2008). For example, in a study on amphibian larvae survival rates, Sih et al. (2004) observed an increased mortality rate in some species to sub-lethal pesticide concentration when predatory cues were also found in the environment, whereas mortality rate of others seemed indifferent to the presence of such cues. Overall, the response of different species to a specific stressor is context-dependent and modulated by both the other species in the community and the presence of additional stressors (Bruder et al., 2017; Lenihan et al.,

2018; Saaristo et al., 2018). Therefore, the outcome of multiple stressors is highly diverse. To illustrate this diversity, a meta-analysis conducted on over 171 studies in marine and costal ecosystems found that the cumulative effects of stressors were additive in 26% of the cases, synergistic in 36% and antagonistic in 38% (Crain et al., 2008). Similar results were also found in freshwater studies (Nõges et al., 2016). Using their extended definition of antagonism and synergism, Piggott et al. (2015d) revisited the numbers from Crain et al. (2008) and found 43% of antagonism, 31% of synergism and 26% of additive interactions. Although the difference is minimal it highlights the need for the ecological community to find a consensus about the best framework to be used to make generalisation (Nõges et al., 2016; Schäfer and Piggott, 2018).

1.3. Ecosystem functioning and multiple stressors

Ecological processes and functioning, such as organic matter (OM) decomposition, one focus of this thesis, are particularly sensitive to abiotic changes (Woodward et al., 2012). OM decomposition can be separated into four distinct processes: non-enzymatic chemical reactions, leaching and volatilization, comminution, and catabolism (Canhoto et al., 2016). In aquatic environments, leaching, comminution and catabolism are the most important drivers of decomposition. Indeed, leaching is a continuous process occurring when water flows through OM resulting in large organic compounds and inorganic ions losses whereas comminution results from physical abrasion (water flow, invertebrate action, freezing/thawing cycles) leading to a particle size reduction. Whilst leaching and comminution can be respectively exclusive and partially passive processes, catabolism is entirely mediated by the biological activity of microbes and invertebrates.

OM decomposition plays an important role in nutrient cycling but also regulates the bottom-up energy transfer between trophic levels (Kuehn et al., 2011; Woodward et al., 2016, 2010). Central to OM decomposition are microbial and macroinvertebrate activities (Kuehn, 2016; Reiss et al., 2010). Indeed, leaf litter decomposition is modulated by microbial activity, via the production of Carbohydrate-Active-Enzymes (CAZymes) (Abdel-Raheem and Shearer, 2002; Canhoto et al., 2016; Romaní et al., 2006; Sinsabaugh and Findlay, 1995). These enzyme are then responsible for the degradation and modification of complex carbohydrates (cellulose and lignin) which condition the leaves prior to invertebrate consumption (Bärlocher, 2016). The ability of microbes to access stored and dissolved nutrients from decomposing OM and water

respectively, thus creates a nutrient rich and easily digestible substrate on which higher trophic levels can easily feed on (Danger et al., 2016).

Many multiple stressor studies in stream systems have studied the combined effects of anthropogenic factors on microbial activity (Fernandes et al., 2014; Kominoski et al., 2015; Manning et al., 2018; Piggott et al., 2015a). Overall, stressors increasing the metabolic rates of microbes tend to enhance the microbial activity and the decomposition of leaf litter. However, one aspect that is less often investigated in such studies is the identity and structure of the microbial community. This potentially could limit our mechanistic understanding of organic matter decomposition under multiple stressors which often depends on microbial community identity and diversity (Gessner et al., 2010). Although the traditional theory stipulates that microbial communities have a high level of functional redundancy (Bell et al., 2005), there is still some debate around the part played by microbial biodiversity and ecosystem functions (Bender et al., 2016; Martínez and Canhoto, 2019; Tolkkinen et al., 2015a) Microbes thus occupy a pivotal role in nutrient and carbon cycling, most notably the energy transfer in the detrital food chain, yet the response of the 'microbial blackbox' to multiple stressors, and its consequences for OM decomposition, carbon and nutrient cycling, needs further evaluations.

1.4. Taxonomic vs functional approaches to understand the effects of multiple stressors

The high diversity of possible outcomes forces multiple stressor studies to investigate a wide range of metrics in order to make sense of this complexity and understand the biological mechanisms behind stressor interactions. Further, differences exist according to the level of taxonomic resolution used (community level, population level, trophic level) (Crain et al., 2008; Nõges et al., 2016). Therefore, in order to understand the interactions between stressors, multiple stressor studies strongly benefit from multi-layered and multitrophic assessment.

Multiple stressor research generally relies on structural response variables such as community composition and diversity measurements (Hering et al., 2006). Although very informative to evaluate the geographical distribution of populations facing different stressors, theses metrics provide little mechanistic insight into the actions of stressors. To improve this issue, a second category of measurements focuses on important ecosystem functions such as decomposition, respiration and primary production (Manning et al., 2018; Niyogi et al., 2003; Piggott et al.,

2015b). However, similarly to community metrics, ecosystem functioning measurements are aggregate features only allowing the overall activity of the community in response to stressors to be evaluated but not necessarily the pathways taken. One way to circumvent this problem is to simultaneously record both community structure and ecosystem functioning measurements (Piggott et al., 2015b). This way, one can directly assess change in community populations and overall structure as well as the matching ecosystem functioning response. However, there are a large number of functions and it is not possible to record all of them.

A very practical and cost-effective way to acquire functional knowledge about communities whilst recording structural changes has been the use of phenotypic trait-based biomonitoring approaches and trait databases (Ding et al., 2017; Hamilton et al., 2019). In this method, the selection of traits to measure usually derives from the consideration of the predictable effects of environmental constraints on biological traits. For instance, invertebrates that specialise in crawling versus swimming need very different sets of morphological adaptations and body structures due to mechanical differences of the two means of locomotion. Similarly, differences in feeding and life-history strategies and can relate to system productivity across trophic levels (Cummins, 2016). From the trait information obtained, a new suite of metrics can be calculated; referred to as functional diversity (Mason et al., 2013; Villéger et al., 2008). Whilst the concept of functional diversity has more often been applied to terrestrial environments (Mason and Pavoine, 2013), it can also be very useful to stressor research in revealing community assembly processes along a stressor gradient (Wu et al., 2019). Indeed, functional diversity measurements can link with stability, resistance and resilience of ecosystems to perturbations (Bruno et al., 2016). Understanding the stability patterns of communities can greatly improve our ability to implement successful stressor mitigation strategies, and therefore functional diversity measurement ought to occupy a more important place in stressor research. Trait and functional diversity approaches have the advantage of freeing our assessment of the effect of multiple stressors from species identity across temporal and spatial scales (Bêche et al., 2006; Menezes et al., 2010; Poff, 1997) and brings insight into the mechanisms underlying effects of stressors (Menezes et al., 2010; Statzner and Bêche, 2010; Townsend and Hildr, 1994). Indeed, trait responses to environmental variables highlight the functional significance of species emphasizing on the filtering role that stressors have on communities. Traits are also applicable to any locality, therefore trait responses from completely different places, and thus most likely from completely different taxonomic pools, can still be compared with no bias.

1.5. Considering the spatial and temporal context in multiple stressor assessments

A central concept in ecological research is the environmental filtering concept where abiotic conditions select for species that are the most adapted to them (Poff, 1997). Stressors can indeed trigger spatially organised taxonomic and functional community shifts, modifying the functioning of ecosystems (Li et al., 2019). However, our interpretations of stressor effects on biodiversity are likely to be influenced by spatial and temporal patterns of both communities and stressors themselves (Harvey et al., 2019, 2018; Harvey and Altermatt, 2019). Indeed, the local community assembly of species depends on the regional taxon pool, the dispersal limitation of species, local habitat conditions and biotic interactions (Schuwirth et al., 2016). Thus, change in spatial abiotic heterogeneity influences the persistence of sensitive species over time if they are unable to find new suitable conditions (Tonkin et al., 2018). Further, communities are not only spatially connected but also temporally. Thus, there is a feedback loop created with communities resulting from past abiotic conditions influencing future biodiversity responses (Baumgartner and Robinson, 2015). Finally, stressor temporal regimes, i.e. their frequency, order of occurrence and time of exposure also influence the biodiversity responses observed (Davis et al., 2019). Indeed, taking the example of stressor frequency, chronic exposure does not allow the recovery of communities between events and therefore the biodiversity response will change between the two observations. All of the above biodiversity spatiotemporal considerations have a significant impact on how we perceive the effect of multiple interacting stressors and how we address them. They modify the stability of ecosystems under stress and as a result of biodiversity changes, important ecological functions may be lost or altered (Tilman and Downing, 1994).

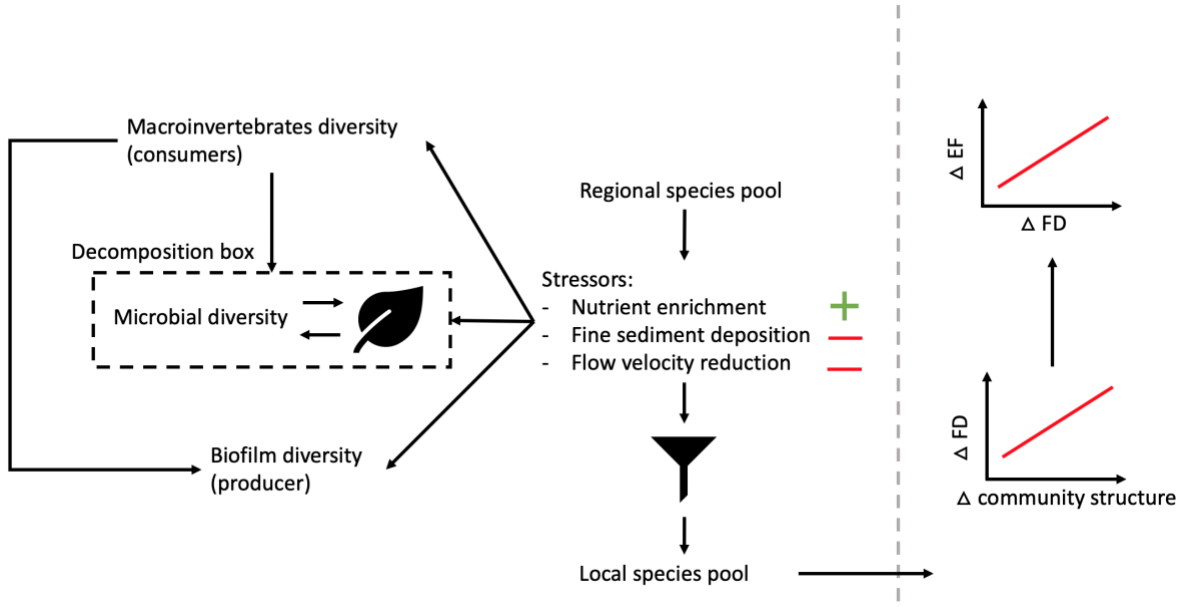


Figure 1.1: PhD project conceptual model. From the species pool of our studied stream, we apply a filtering effect with the different stressors combinations which then influences the taxonomic and functional diversity (FD) of our three trophic levels (decomposers, producers and consumers) via identity and community structure changes. Ultimately this is reflected in the variations of the ecosystem functioning (EF). The different trophic levels are connected via feeding interactions where the decomposer microbes process the leaf litter and then the macroinvertebrates feed on both the decomposition litter and the biofilm.

1.6. Thesis outline and aims

This thesis aims to investigate the individual and interactive ecological responses of benthic communities, populations and functions to abiotic land-use stressors, which co-occur and dominate lotic ecosystems worldwide; namely nutrient enrichment, sedimentation and flow-velocity reduction. The research will evaluate the frequency of stressor interactions between different trophic levels, their directionality and the effect of the length of exposure to the stressors. To address these aims we conducted three simultaneous mesocosm experiments looking at microbial decomposers, microbial biofilm and macroinvertebrates in a unique flow-through streamside field system. Each experiment targeted a critical layer of lotic ecosystem health and functioning which together can assist decision-makers in making holistic assessments of stream and river ecological responses to changing environments (Fig 1.1). Because each experiment occurred simultaneously in the same experimental units, the results are directly comparable giving an overall assessment of the whole-system response to the applied treatments. The data chapters are formatted in a paper-based style suitable for journal submission.

Chapter 2 – In headwater streams leaf litter input represents an important energy source to stream communities, providing nutrients and carbon supply to food webs, and modulating species dynamics. Microbial activity is a key process within the organic decomposition performed by stream ecosystems by making leaf litter substrates more labile to higher trophic levels but also by rendering resources that would otherwise remain trapped in cellulosic plant tissues accessible. Microbial activity and communities are known to be sensitive to environmental parameters, however little is known about their successional patterns under a multiple stressor scenario and the resulting impact on ecosystem function. In this chapter I used molecular techniques to investigate the single and combined effects of nutrients, sedimentation and flow velocity on bacterial and fungal communities associated with leaf litter decomposition. I addressed the following questions: (1) *Which of bacterial and fungal communities are more sensitive to stressor combinations?* (2) *Is time of exposure to stressors an important parameter to be considered?* (3) *Are the community patterns observed transcribed into the efficiency of leaf litter decomposition?*

Chapter 3 – Bacterial biofilms have a central position in stream ecosystems, often being considered as the "skin" of rivers. Amongst others, biofilms are involved in the bioremediation of pollutants, nutrient cycling, essential ecosystem processes (whole respiration), but also provide an important food source to higher trophic levels such as macroinvertebrates for instance. Moreover, bacterial community assemblages result from a trade-off between filtering forces selecting for the most adapted species and evolutionary interaction forces such as competition, facilitation or inhibition. Despite their key positioning in the functioning of lotic ecosystems and trophic networks, our understanding of bacterial biofilm community dynamics under multiple stressor scenarios remains limited. Most notably, there is a lack of characterisations of community structure and how changes can be used to anticipate far-reaching stream-wide changes in ecosystem functioning. In the chapter we also used molecular techniques to characterise bacterial community responses to interacting stressors, but also explored how population dynamics and assembly structure could be used to monitor stream functioning. We addressed the following questions: (1) *Can groups of bacteria be isolated for the biomonitoring of multiple stressor interactions?* (2) *Can the population dynamics be extrapolated to stream-wide ecosystem processes?*

Chapter 4 – In Chapter 4, the study progresses from the basal trophic-level responses investigated in chapters 2 and 3 to the next trophic level – the macroinvertebrates. Indeed,

macroinvertebrates feed on both decomposing litter and biofilm. Macroinvertebrates are also routinely used in stream biomonitoring schemes across the world. Most notably, groups of macroinvertebrates such as Ephemeroptera, Plecoptera and Trichoptera, (EPT) are particularly sensitive to environmental changes and routinely used in biomonitoring programs (Bonada et al., 2006). Recently, there has been a surged in studies combining macroinvertebrates community structure with functional traits, enabling the evaluation of ecological niche breadths between lotic environments (Ding et al., 2017; Dolédec et al., 2011; Mor et al., 2019). However, macroinvertebrate functional diversity, which can be highly valuable for a mechanistic understanding of stream ecosystem functioning and stability, is less often considered and has seldom been assessed in a multiple stressor context. In this chapter I used a combination of taxonomic identification with literature-based functional trait evaluations to answer the following questions: (1) *Are community, taxonomy or trait metrics better suited to evaluate stressors effects and the frequency of interactions?* (2) *How can taxonomy-based and trait-based techniques complement each other to understand the effects of stressor interactions?*

Chapter 5 – In the three previous data chapters we explored several facets of multiple stressor interactions on the integrity of lotic ecosystems. Multiple stressor research is a fairly new field although it has attracted a lot of attention over the last 10 years. Thus, there are many theoretical gaps that need to be explored, most notably to understand the biological mechanisms of stressor interactions. So far, most of the stressor research effort has been placed towards building a database of the responses to diversity and gradients of stressors in various combinations. However, the influence of spatial connectivity, temporal connectivity and biological connectivity in shaping the community response to multiple stressors remains an open field. In this chapter, using our experimental expertise and growing knowledge of multiple stressor research challenges, acquired throughout the PhD program, I propose a vision of how the state of the art can be improved. We explored the following question: *How can integrating spatial and temporal complexity into multiple stressor research further our understanding of stressors interactions?*

Chapter 6 – In this general conclusion chapter I synthesised the findings and reflections of chapters 3-6 to place them in the wider context of multiple stressor research. Specifically, I show how our interpretation of multiple stressor interactions can be biased towards the study system. Based on our results from three distinct trophic levels and from three different ways to

evaluate ecological functions, I explain how multitrophic community assessments advance our understanding of interactions caused by multiple stressors in lotic ecosystems.

2. Chapter 2: Anthropogenic stressors affect fungal more than bacterial communities in decaying leaf litter: a stream mesocosm experiment

2.1. Abstract

Despite the progress made in environmental microbiology techniques and knowledge, the succession and functional changes of the microbial community under multiple stressors are still poorly understood. This is a substantial knowledge gap as microbial communities regulate the biogeochemistry of stream ecosystems. Our study assessed the structural and temporal changes in stream fungal and bacterial communities associated with decomposing leaf litter under a multiple-stressor scenario. We conducted a fully crossed 4-factor experiment in 64 flow-through mesocosms fed by a pristine montane stream (21 days of colonisation, 21 days of manipulations) and investigated the effects of nutrient enrichment, flow velocity reduction and sedimentation after 2 and 3 weeks of stressor exposure. We used high-throughput sequencing and metabarcoding techniques (16S and 18S rRNA genes) to identify changes in microbial community composition. Our results indicate that (1) shifts in relative abundances of the pre-existing terrestrial microbial community, rather than changes in community identity, drove the observed responses to stressors; (2) changes in relative abundances within the microbial community paralleled decomposition rate patterns with time; (3) both fungal and bacterial communities had a certain resistance to stressors, as indicated by relatively minor changes in alpha diversity or multivariate community structure; (4) overall, stressor interactions were more common than stressor main effects when affecting microbial diversity metrics or abundant individual genera; and (5) stressor effects on microbes often changed from 2 weeks to 3 weeks of stressor exposure, with several response patterns being reversed. Our study suggests that future research should focus more on understanding the temporal dynamics of fungal and bacterial communities and how they relate to ecosystem processes to advance our understanding of the mechanisms associated with multiple-stressor interactions.

2.2. Introduction

Microbial communities drive stream biogeochemistry by playing a crucial role in ecosystem processes, such as ecosystem respiration, organic matter decomposition and nutrient cycling (Bruder et al., 2016a; Kuehn, 2016; Manning et al., 2018). For example, microbial communities fix nutrients onto substrata and improve the palatability and quality of decomposing leaf litter, thus providing a high-nutrition food source for higher trophic levels (Kuehn, 2016). Their influence therefore starts at the bottom of the food chain, and then potentially shapes higher trophic-level communities along the river continuum. One of the central aims of microbial ecology is to understand how environmental changes drive the structure and function of communities (Herren et al., 2016). In ecology, 'stressors' can be defined as any anthropogenic activity resulting in environmental change that will take the studied system outside of its normal operational range (Sabater et al., 2019). Studies have previously revealed that microbial communities follow successional stages; however, the forces driving this progression are less well understood (Knelman et al., 2014). Indeed, community responses to environmental changes can either be stochastic or deterministic. Therefore, if the latter is true, then the microbial community's adaptation to stress can be predicted, always selecting for the species that are most adapted to the new conditions. However, the high functional redundancy within microbial species in a community (Bell et al., 2005) may lead to stochastic responses limiting the replicability of the observed patterns.

Stressors associated with agriculture and urbanization, for example sedimentation, reduced flow velocity and nutrient enrichment, are known to have significant effects on microbial assemblages, productivity and activities, and thus can lead to changes in ecosystem function such as altered decomposition rates (Pascoal and Cássio, 2004; Piggott et al., 2015a; Widder et al., 2014). Individually, sediment deposition and flow velocity reduction influence microbial community structure and diversity by creating physical barriers via an increase in boundary layer thickness (Barker Jømgensen and Des Marais, 1990; Stevens and Kurd, 1997) or a filtering action of the hyporheic zone (Cornut et al., 2014). Both processes limit the exchange of oxygen and nutrients between water column and biofilm but also modulate the dispersal-colonisation dynamics of the substratum (Besemer et al., 2007; Cornut et al., 2014). The effects of nutrient enrichment on litter-associated microbial communities are complex and can differ depending on microbial stoichiometry (Brosed et al., 2017), nutrient uptake rates (Gulis and Suberkropp, 2003), substratum stoichiometry (Manning et al., 2016), the environment's

reference conditions and enrichment magnitude (Ferreira et al., 2015). In general, however, moderate nutrient enrichment tends to promote microbial growth and activity.

Stressor effects are known to vary along disturbance gradients, sometimes taking unforeseen directions regarding the microbial community's structure and function (Romero et al., 2019b). For instance, stressors leading to changes in the physico-chemical (water chemistry, flow velocity, benthic substratum composition) and biological conditions along the river continuum are known to alter fungal presence, traits, life strategy and decay activities (Kuehn, 2016). Further, fungi and bacteria both co-habit the microbiome and play key roles in the decomposition of organic matter. During this process, complex biotic interactions between the two organism groups can occur. While it is generally agreed that both fungi and bacteria can influence each other's community structure, the questions of whether their interactions are predominantly facilitative or competitive, and how these interactions translate into ecosystem function, are still open and also context-dependent (Frey-Klett et al., 2011; Johnston et al., 2016; Romaní et al., 2006).

There has been some interest in the mechanisms which drive the spatial and temporal microbial community changes in response to stressors, including the interactions between bacteria and fungi. Several studies in terrestrial and aquatic environments have highlighted inter- and intra-kingdom interactions and dynamics (Gessner et al., 2010; Purahong et al., 2016). Nevertheless, how stressors may perturb these dynamics has been assessed less often in streams. Further, incubation time has also been shown to be a strong determinant of microbial community structure (Newman et al., 2015). Indeed, as decomposition advances, more recalcitrant compounds remain, favouring the development of microbial species capable of metabolising such compounds (Gessner et al., 2010). Stressors and stressor combinations have the potential to disrupt these processes by accelerating or slowing down decomposition rates as well as altering microbial succession and activity. Thus, a clearer understanding of these processes requires temporal studies at a fine taxonomical resolution for both bacteria and fungi.

Understanding and making sense of the microbial diversity and functionality under stress is a hard task to implement. Indeed, manipulative experiments on multiple stressors require considerable logistical investment to set up, and much of the research linking bacteria and fungi to litter decomposition in freshwater systems has been conducted on sterile (autoclaved) substrata in laboratory settings (Fernandes et al., 2014; Ferreira and Chauvet, 2012; Gulis et

al., 2017). However, only a few microbes can be successfully reared and identified in laboratory conditions (Lloyd et al., 2018; Steen et al., 2019). Therefore, these studies provide a very simplified version of the natural environment (Johnston et al., 2016). Further, leaves falling into streams already come with their own set of microbes, as either endophytes (leaf interior) or phyllosphere (leaf surface) organisms, and several aquatic microbial genera are known to have a terrestrial stage in their life cycle (Mustonen et al., 2016; Röhl et al., 2017). Nevertheless, past research tends to overlook this initial community and how it could influence the successional trajectory of the microbial community during the leaves' aquatic decomposition. Recent advances in molecular techniques have enabled deeper investigation of the microbial "black box" in its natural environment (Cristescu, 2014). However, understanding global change effects on microbial communities and the resulting change in ecosystem function is still hindered by a lack of characterisation of communities (Antwis et al., 2017). Thus, questions about adaptation, functional redundancy, stochastic versus deterministic assembly patterns and temporal variations have been identified as some of the most prominent open questions in microbial ecology (Antwis et al., 2017).

Here we used field mesocosms to investigate the influence of nutrient enrichment, flow velocity reduction, increased sedimentation and their interactive effects on the microbial communities associated with decomposing leaf litter along a temporal gradient of three weeks. Through investigating the temporal dynamics of bacterial and fungal microbes, we aimed to show that stressor accumulation disrupts the natural microbial successional patterns, resulting in changed organic matter decomposition rates. We tested four specific hypotheses:

(1) By providing readily available extra resources, nutrient enrichment will enhance decomposition rates and enhance microbial diversity (Gulis and Suberkropp, 2004; Kerekes et al., 2013; Piggott et al., 2015a).
(2) Flow velocity reduction will slow decomposition rates and change microbial community composition, due to an increase in boundary layer thickness reducing O_2 availability and nutrient availability (Barker Jømgensen and Des Marais, 1990; Bruder et al., 2016a; Stevens and Kurd, 1997).
(3) Fine sediment will also decrease decomposition rates, by changing the microbial community's functionality (e.g. by reducing efficiency in enzymatic activity of change in metabolic pathways; Tank et al., 2013).
(4) The positive nutrient effect on decomposition and microbial diversity will be counteracted by sedimentation and flow velocity reduction because both act as a

physical barrier limiting resource exchange between microbiome and water column, resulting in antagonistic two-way and three-way interactions.

2.3. Methods

2.3.1. Study site

Our study occurred in a streamside mesocosm setup (ExStream System) fed by the Yinxi Stream originating from the Jiulongfeng Nature Reserve, Anhui Province, China. The mesocosm site is located just downstream of the reserve boundary (30°07'07''N, 118°01'24''E, 330 m a.s.l.). The reserve covers a 2720 ha area (98% forested, 80% native vegetation) on the west side of the main Huangshan Mountain massif. The reserve's vegetation distribution displays an altitudinal gradient of evergreen broad-leaved forest, evergreen deciduous broad-leaved forest, deciduous broad-leaved forest, alpine short forest and alpine meadows (Jiulongfeng Nature Reserve Director, Mr. X. H. Cao, personal communication). Mean annual temperature and precipitation are 15.4°C and 1500-1600 mm, respectively (Huangshan District Government, http://www.hsq.gov.cn/; accessed August 2019).

Yinxi Stream is a near-pristine montane stream ($N\text{-}NO_3^-$ 0.39±0.008 [SE] mg/L, $N\text{-}NH_4^+$ 0.26±0.002 mg/L, $P\text{-}PO_4^+$0.01±0.001 mg/L, pH 7.87±0.018, conductivity 46.45±0.029 SE µS/cm; four measurements each collected with a YSI (Professional Plus, YSI Incorporated, Yellow Springs, OH, USA) at the mesocosm system's water intake point on September 24th 2018). The stream is bordered by steep slopes with dense forest shading the streambed. The only human impact on the stream is a small hydrologic dam located approximately 2.8 km upstream.

2.3.2. ExStream system and experimental design

The experiment ran for 42 days from October 1st to November 12th 2018. Since allochthonous carbon inputs can play an important role in stream dynamics (Gounand et al., 2018b), the experimental period was chosen to include one autumn leaf senescence event to maximise microbial diversity and productivity.

The study was conducted using a 64-unit outdoor stream mesocosm system (ExStream Systems Ltd., Dunedin, New Zealand, Fig. 2.1) similar to mesocosms setups previously used in New

Zealand (Piggott et al., 2015c), Germany (Elbrecht et al., 2016) and Ireland (Davis et al., 2018). Briefly, water and the associated drifting aquatic invertebrates, algae and microbes from the Yinxi Stream were continuously pumped (ACm150B2, Leo Group Co., LTD, Zhejiang, China) through a 4-mm mesh filter into four header tanks, each of which gravity-fed 16 circular mesocosms (outer diameter 24.5 cm, inner diameter 5.1 cm, volume 3 L, area 450 cm^2; Microwave Ring Moulds, Interworld, Auckland, New Zealand). The experiment comprised a 21-day colonisation period followed by a 21-day manipulation phase.

We used a full factorial 2×2×2 design with eight replicates for each stressor combination: nutrients (ambient versus increased N-NO_3^- and P-PO_4^{+}), flow velocity (control versus reduced) and fine sediment (control versus added). To assess temporal variation in the microbial responses, the system was sampled on two occasions (Days 14 and 21). On each occasion, 32 mesocosms from two randomly selected header tanks were sampled (four replicates per treatment combination). Each mesocosm was sampled only once during the experiment.

The water flow through each mesocosm was maintained at a constant rate of 2 L/min and recalibrated daily. Water leaving the mesocosms flowed through their inner circular opening, allowing natural emigration of stream invertebrates and microbes by drift. Temperature and light intensity were monitored every 5 min in one randomly selected mesocosm per header tank block using a HOBO pendant MX2202 data logger (Onset, USA). Mean water temperature over the length of the experiment was 16.14 ± 2.13 [SD] °C compared to 15.60 ± 1.72 [SD] °C at the pump intake in the river. Each mesocosm received 500 mL of coarse substratum (> 2 mm), ten 3-4 cm surface stones and one large stone (> 6 cm). This substratum composition represented similar habitat heterogeneity as reported in Chinese streams and rivers (Liu et al., 2016). The substratum was collected from a nearby, dry floodplain section of the Yinxi Stream.

Leaves from *Cinnamomum camphora* (camphor tree) were collected from a single street stretch in Suzhou, Jiangsu, China (31°16’17.96’’N, 120°44’33.86’’E) and air-dried in the laboratory for at least 2 weeks before being stored in the dark. *C. camphora* is an evergreen tree endemic to the southern Yangtze regions and one of the most widespread tree species across China. It is commonly used as an ornamental tree in cities and rural areas and is also being found near our study area (N.P.D. Juvigny-Khenafou, personal observation). Leaf bags consisted of 2.5 ± 0.01 [SD] g of dried leaf material placed into 4-mm mesh bags. To each mesocosm, two leaf

bags containing dried *C. camphora* leaves were added on Day-7 to allow microbial colonisation. Leaf bags were pinned to the side of the mesocosms and kept flat on the substratum using surface stones, similar to real-life situations when leaves get trapped under surface rocks. An additional 5 g of the original litter, hereafter referred as terrestrial litter, was also stored at –20 °C to determine the original microbial community present in and on the leaves before the experiment started (Röhl et al., 2017).

Water flow and drift colonisation of the mesocosms started on 1 October 2018 (Day-21). On Day-4, macroinvertebrates were collected from the Yinxi Stream upstream of the pump intake from eight similar riffle environments using kick-net sampling for 3 min of a ~0.36 m_2 area (comparable to the benthic surface area of eight mesocosms). These invertebrates were added to the mesocosms to supplement natural colonisation by taxa underrepresented in the drift (Elbrecht et al., 2016; Piggott et al., 2015b). Following collection, each kick-net sample was divided into eight equal portions using a subsampler and then randomly distributed to individual mesocosms (one portion per mesocosm) following Elbrecht et al. (2016).

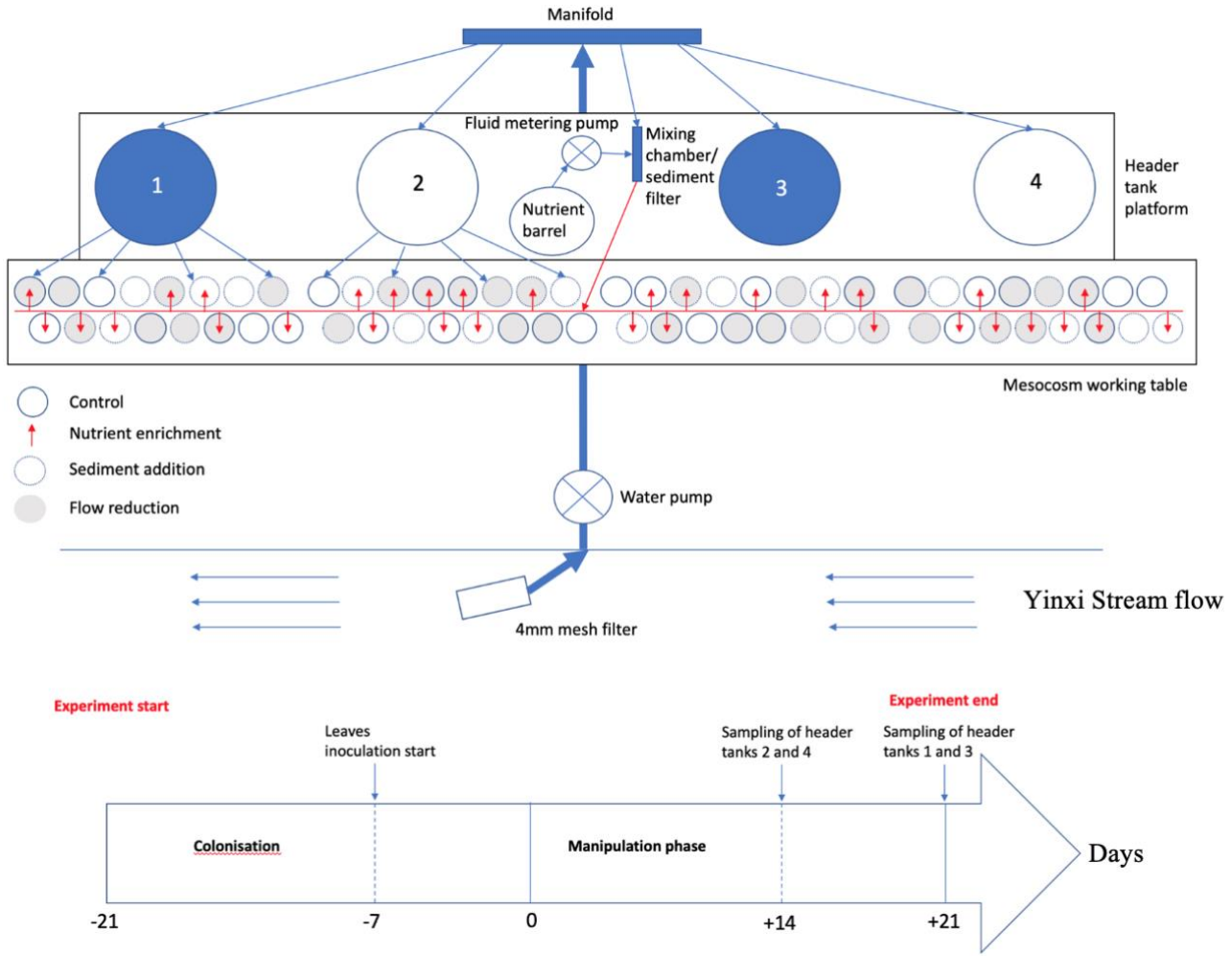


Figure 2.1: Schematic of the experimental design and timeline of the experiment.

2.3.3. Stressor manipulation

Stressor combinations were randomly assigned within each block on 22nd October 2018 (Day-0). For the sediment treatment, flow in 32 mesocosms was interrupted for 5 min and 300 mL of fine sediment were added. This sediment had been collected from a dry floodplain downstream of the system, air-dried for one week and then sieved (mesh size 0.5 mm, D_{50} = 411.6 μm, Bettersize BT-2900, China) prior to addition. This treatment resulted in 100% sediment cover of the mesocosm substratum on Day-1, consistent with high sediment cover levels observed in catchment-scale stream and river surveys in China (Liu et al., 2016). However, due to the thickness of the leaf bags only the topmost leaves were fully covered by fine sediment, similar to leaves piling up and anchored by a few surface stones in real streams.

For the flow velocity reduction treatment, the inflow jets were removed and the inlet pointed downwards in 32 mesocosms, to decrease flow velocity whilst keeping identical discharge. This avoided confounding effects on nutrient concentrations and on unmanipulated physicochemical (e.g. water temperature, dissolved oxygen) and biological variables (e.g. drift of stream biota). Near-bed flow velocities in all mesocosms were recorded weekly (Days -20, -14, -5, 3, 10, 18) using an electromagnetic flow meter (MF Pro, OTT HydroMet GmbH, Germany). Achieved velocities were 0.10 ± 0.008 [SE] $m.s^{-1}$ in the control treatment and zero (below the instrument's detection limit) in the reduced velocity treatment. Similar near-bed flow velocities have been obtained in previous experiments using the same mesocosm system in other countries (New-Zealand and Germany), and resulted in considerable differences for the measured biological response variables (Beermann et al., 2018a; Bruder et al., 2016a; Elbrecht et al., 2016).

Nutrient enrichment was achieved by continuously injecting a concentrated solution of $NaNO_3$ and KH_2PO_4 into 32 mesocosms using a fluid-metering pump (CK15, Kamoer, Shanghai, China). Nutrient concentrations were determined on Days 1, 8, 15 and 18 (n = 192, only 32 mesocosms remained on the last two dates) using standard methods (APHA, 1998). Sample aliquots were analysed with a Lachat flow injection analyser (QuickChem 8500, Hach, USA). Achieved concentrations were 2.19 ± 0.09 [SE] mg/L $N\text{-}NO_3^-$ and 0.12 ± 0.005 mg/L $P\text{-}PO_4^{+}$ in the enriched treatment compared to 0.57 ± 0.02 [SE] mg/L $N\text{-}NO_3^-$ and 0.01 ± 0.001 [SE] mg/L $P\text{-}PO_4^{+}$ in the ambient treatment. Enrichment levels were chosen to remain in the enriched water quality category according to the 6-class water quality classification (GB 3838-

2002) of the Ministry of Environmental Protection of the People's Republic of China (MEP, 2002), while also representing recognisably enriched levels according to other countries' frameworks (e.g. European Environment Agency, 2015). Further, they represented realistic enrichments in Chinese waterways following agricultural intensification; between 1970 and 2000 TDN and TDP increased 8- and 22-fold, respectively, at the river basin scale (Strokal et al., 2016).

2.3.4. Leaf bag processing

On Days 14 and 21, leaf bags were collected carefully, placed into individual ziplock bags and kept on ice before being frozen at -20°C within 2 hrs of collection. Back in the laboratory, one leaf bag per mesocosm was thawed overnight at 2°C and gently rinsed under running deionised water to remove sediments, invertebrates and other organic matter debris (Graça et al., 2005). All leaf materials were then freeze-dried for 48 hrs in sterile tubes and weighed to the nearest 0.001 mg to estimate the mass loss (Gessner, 1991) before being sent to Sangon Biotech Co., Ltd, (Shanghai, China) for downstream processing.

2.3.5. DNA extraction, PCR amplification and sequencing

The whole content of each leaf sample was homogenised in liquid nitrogen and 250 mg of the material was used for DNA extraction using the Mag-Bind Soil DNA Kit (Omega E.Z.N.A.™, Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. All samples were later processed by next-generation sequencing (Illumina MiSeq) using the PCR primer pairs (forward/reverse) 341F/805R (Du et al., 2018) and NS1/GCfung (Maza-Márquez et al., 2016), targeting the V3-V4 region of bacterial 16S rRNA and the 5' end of the eukaryote 18S rRNA, respectively. Both forward and reverse primers were tagged with adapter sequences, pad and linker regions and a unique barcode on the forward primer to permit the multiplexing of samples. All primers were provided by Sangon Biotech Co., Ltd., Shanghai, China. Nested PCRs were performed, following Du et al. (2018), and the samples were prepared for sequencing using a TruSeq DNA kit according to the manufacturer's instructions. The PCR-amplified products were examined by agarose gel-electrophoresis, purified using Agencourt AMPure XP beads (Beckman, Brea, CA, USA) and quantified with the Qubit 3.0 DNA test kit (Life Technologies, Carlsbad, CA, USA). Amplicons were then pooled in equimolar concentrations in the final mixture. The libraries were sequenced at 20 pmol/µL on MiSeq, 2

× 300 bp paired-end version 3 chemistry according to the manufacturer's specifications by Sangon Biotech Co., Ltd, Shanghai, China.

Sequences were processed and analysed using QIIME 1.8.0 based on sequence length, quality, primer and tag. The forward and reverse reads were joined with an overlap length of 150 bp. Following removal of the primer, all reads having a tail quality score below 20 (with a 10 bp window), containing ambiguous characters and less than 200 bp were removed. Chimeras were identified using the UCHIME software (Edgar et al., 2011). We did not rarefy the samples because of the associated issues concerning sample richness comparability (Chao and Jost, 2012; McMurdie and Holmes, 2014). The filtered reads were then clustered into OTUs (Operational Taxonomic Units) with USEARCH using a 97% similarity (Edgar, 2010) and OTUs found in only one read across the dataset were discarded. The average length of the remaining filtered sequences was 412 bp and 422 bp for the prokaryote and eukaryote dataset, respectively. The taxonomic assignment of OTUs was performed against the Silva database with the Ribosomal Database Project classifier and a minimal confidence score of 0.8 (Gustave et al., 2019; Purahong et al., 2016; Xiao et al., 2018). Coverage estimators were calculated for each sample to ensure their comparability prior to downstream analyses (Chao and Jost, 2012; Shimadzu, 2018). Raw prokaryotic and eukaryotic data were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA560484.

2.3.6. Statistical analysis

All statistical analyses were performed using R (version 3.5.2, R Core Team). The model structure used for all analyses (unless stated otherwise) was the following: intercept (d.f. 1) + nutrients (1) + sediment (1) + velocity (1) + time (1) + nutrients x time (1) + sediment × time (1) + velocity × time (1) + nutrients × sediment (1) + nutrients × velocity (1) + sediment × velocity (1) + nutrients × sediment × time (1) + nutrients × velocity × time (1) + sediment × velocity × time (1) + nutrients × sediment × velocity (1) + nutrients × sediment × velocity × time (1) + error (48; n = 64).

The significance level was set at $p < 0.05$, and all response patterns summarised in the Results were significant unless indicated otherwise. Standardised effect sizes (partial η^2 values, range 0-1; Garson, 2015) are presented for all p-values < 0.1 to allow our readers to evaluate the likely biological relevance of the results (Nakagawa, 2004), except for the PERMANOVA (see

below). Following Nakagawa and Cuthill (2007), effect sizes can be classified as: <0.10 'very small', ≥0.10 'small', ≥0.30 'medium', and ≥0.50 'large'. Since all but two of the significant higher-order interaction terms had smaller effect sizes than the corresponding significant lower-order and/or main effect terms (see Results), the latter could mostly be interpreted reliably (Quinn and Keough, 2002).

We first generated relative abundance. When investigating taxon-specific response patterns, we adopted a slightly more conservative approach (genus level) than the OTU (species) level. This taxonomic resolution adopted the middle ground between the recommendations of a previous mesocosm study in a similar experimental system in New Zealand, which revealed that a resolution below order did not necessarily generate more accurate results when detecting stressor interactions (Salis et al., 2017), and most other recent microbial multiple-stressor studies which focused on OTUs. Bacterial and fungal alpha diversity, richness, Shannon index and Pielou's evenness were computed and analysed with the linear model detailed above. To investigate the total community response (including rare taxa) to the stressors, a PERMANOVA analysis (Bray-Curtis coefficient and 999 permutations) was performed, and a permutational analysis of multivariate dispersions (PERMDISP; Anderson, 2006) conducted to assess homogeneity. Since the bacterial community did not respond to any of the treatments or interaction combinations in the PERMANOVA, Principal coordinate analysis (PCoA) plots were made for the fungal community only to allow visual representation of significant PERMANOVA outputs. PCoA plots were based on Hellinger-transformed data to reduce the weights for genera with low read counts and to allow subsequent Euclidian representation of data based on Euclidian distances (Legendre and Gallagher, 2001). MANOVAs were then performed on the abundant fungal and bacterial taxa (>1% of the total reads, Baltar et al., 2015) to investigate taxon-specific responses. All multivariate analyses were performed using the R base functions and the *vegan* package (Oksanen et al., 2015).

2.4. Results

2.4.1. Stressor and time effects on leaf decomposition

Nutrient levels, flow velocity and exposure time all affected leaf decomposition (Fig. 2.2, Table 2.1); mean leaf mass loss across all treatments was 34% (range 28-42%) and 37% (31-47%) after two and three weeks, respectively. Leaf mass loss was higher in nutrient-enriched mesocosms and lower at slower flow, with similar response patterns after two and three weeks

of incubation. No 2-way, 3-way or 4-way interactions between experimental factors were detected.

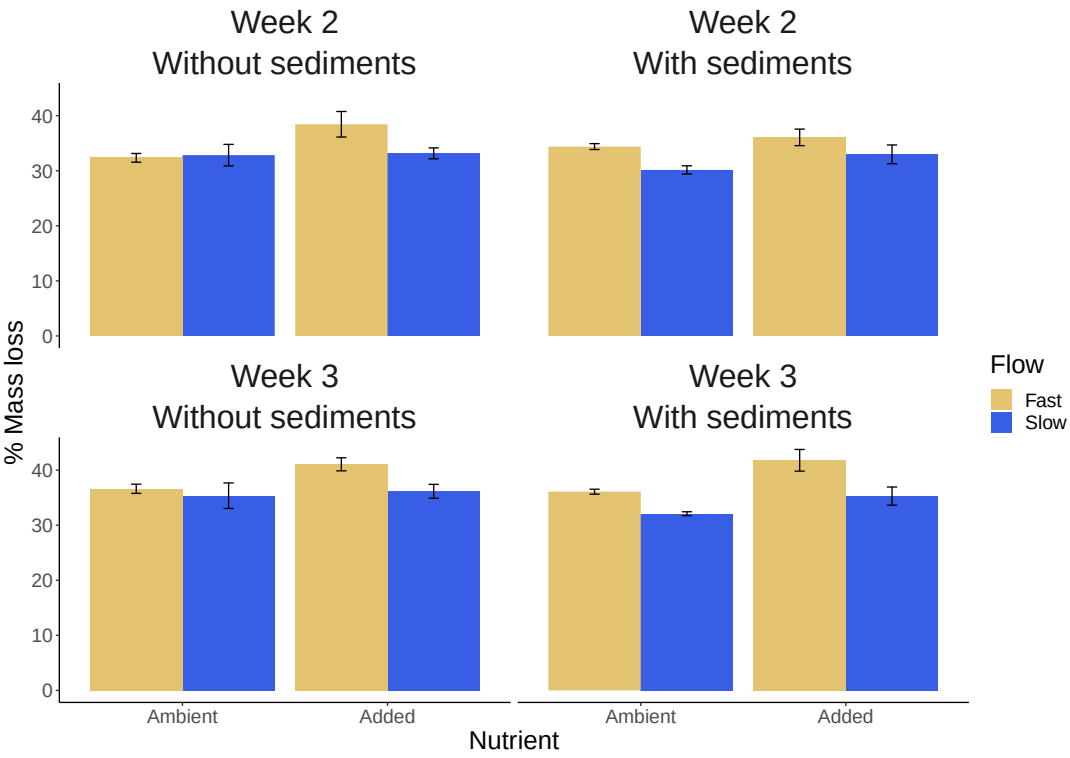


Figure 2.2: Mean percentages of leaf litter mass loss (with standard errors) in the experimental treatments after 2 and 3 weeks of incubation.

Table 2.1: Summary (p-values and effect sizes) of LM results comparing microbial alpha diversity and leaf litter mass loss. For all manipulated factors, main effects are classified directionally as positive (+) or negative (–). P-values bolded where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.1$.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Nutrient X Time	Sediment X Flow	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Bacteria															
Richness	0.34	0.20	0.62	0.93	0.07 (0.06)	0.09 (0.06)	0.94	0.41	0.62	0.55	0.052 (0.08)	0.98	0.94	0.89	0.84
Shannon's	0.73	0.98	0.81	0.99	**0.049** (0.08)	0.58	0.89	0.97	0.72	0.32	0.41	**0.02** (0.10)	0.41	0.31	0.06 (0.07)
Pielou's	0.88	0.74	0.67	0.97	0.09 (0.06)	0.88	0.88	0.79	0.79	0.36	0.71	**0.01** (0.12)	0.39	0.24	**0.03** (0.09)
Fungi															
Richness	**0.001** (0.19) +	0.40	0.19	0.27	0.66	0.48	0.09 (0.06)	0.22	0.25	0.17	0.40	0.15	**0.04** (0.08)	0.77	0.51
Shannon's	0.08 (0.06)	0.83	0.08 (0.06)	0.34	0.12	0.47	0.13	0.54	0.74	0.06 (0.07)	0.58	0.20	0.14	0.30	0.053 (0.07)
Pielou's	0.32	0.97	0.11	0.46	0.10	0.52	0.20	0.29	0.44	0.08 (0.06)	0.39	0.30	0.29	0.27	**0.04** (0.08)
% Mass loss	**<0.001** (0.28) +	0.21	**<0.001** (0.34) –	**<0.001** (0.27) +	0.76	0.06 (0.05)	0.23	0.57	0.9	0.42	0.16	0.33	0.78	0.75	0.31

2.4.2. Microbial community composition in the different microhabitats post treatment exposure

The dataset had a total of 6,360,820 raw reads, 3,422,391 for prokaryotes and 2,938,429 for eukaryotes. Amongst the prokaryote and eukaryote datasets, a respective 2,956,912 and 2,025,927 reads were assigned to 23,141 bacterial and 7,792 fungal OTUs after dereplication, quality filtering and sorting. The Coverage estimator showed that similar degrees of completeness were achieved (16S Coverage range 0.97 - 0.99; 18S Coverage range 0.98 - 0.99) with sufficient sampling depth (16S: 45,491 ± 7,636 [SD]; 18S: 39,257 ± 1,643 [SD]) in each sample.

The post stressor exposure fungal community was dominated by organic-matter-decomposing Ascomycota (99.7% of all reads), amongst which Leotiomycetes (28.3% after 2 weeks of stressor exposure, 33.4% after 3 weeks), Sordariomycetes (28.4%, 20.2%) and Dothideomycetes (25.1%, 27.7%) were the most common classes. Changes in fungal community composition at the class level occurred across all treatment combinations, especially after 2 weeks of stressor exposure (Fig. 2.3).

The post stressors exposure bacterial community was dominated by Proteobacteria (83.3% of all reads), amongst which Alphaproteobacteria (28.8% after 2 weeks, 25.3% after 3 weeks), Betaproteobacteria (27.6%, 31.7%), Deltaproteobacteria (3.5%, 4.3%) and Gammaproteobacteria (22.6%, 22.1%) were the most abundant taxa (Fig. S2.1). At the class level, bacterial communities showed no clear patterns of change in response to the experimental treatments. Community change patterns at the genus level are presented in Section 3.4.

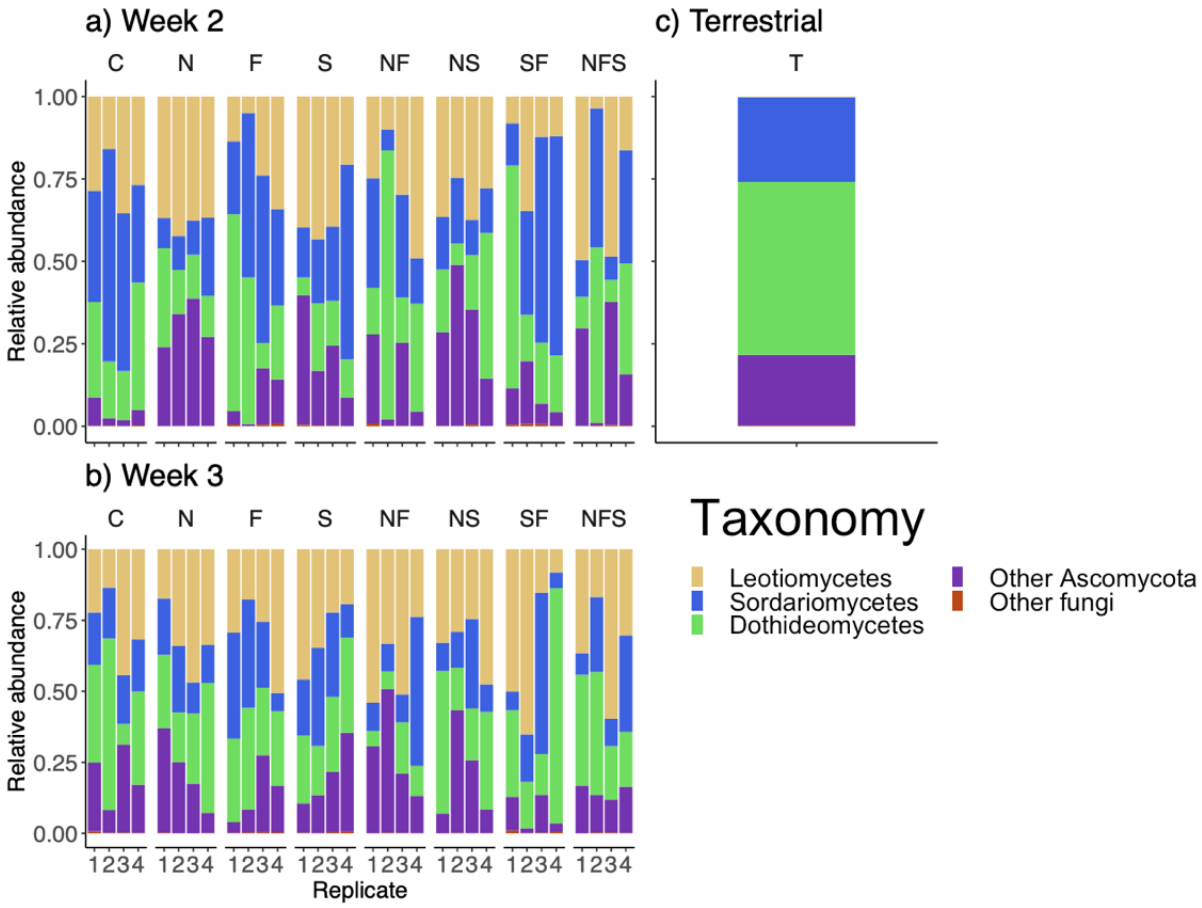


Figure 2.3: Relative abundances of the fungal assemblages at the class level after (a) 2 weeks and (b) 3 weeks; (c) is the terrestrial fungal community before submersion. C: Control; N: Nutrient enrichment; F: Flow velocity reduction; S: Sediment addition; NF: Nutrients + Flow; NS: Nutrients + Sediment; SF: Sediment + Flow; NFS: All three stressors.

2.4.3. Alpha diversity patterns

Diversity metrics for bacteria and fungi responded relatively weakly to the stressors (Table 2.1; see Supplementary Material for bar graphs and 2-way interaction plots for all diversity metrics). Bacterial diversity and evenness showed a nutrient enrichment × sediment addition interaction which changed across sampling dates (Table 1, Fig. S2.4, Fig. S2.6.1-2). After two weeks of incubation, both diversity and evenness decreased with added sediment in nutrient-enriched mesocosms, but this pattern was reversed after three weeks (Fig. S2.6.1-2). Further, evenness displayed a 4-way interaction between all manipulated factors (Fig. S2.4). After two weeks, evenness increased slightly when only sediment was added. This increase turned into a decrease when sediment addition was combined with flow velocity reduction; however, this negative effect of reduced velocity was moderated when nutrients were also added. After three weeks, sediment addition reduced evenness compared to control treatments, but this negative

effect was mitigated by flow velocity reduction and further alleviated when both flow velocity was reduced and nutrients added.

For the fungal community, genus richness generally increased when nutrients were added, and a nutrients × flow × time interaction occurred (Table 2.1). After two weeks, the marked increase in richness associated with nutrient enrichment was weakened somewhat when combined with flow velocity reduction (Fig. S2.5, Fig. S2.6.3-4). After three weeks, richness increased much less with nutrients, and mainly at reduced flow velocity. Diversity was unaffected by the manipulated factors, and evenness displayed a 4-way interaction between all factors (Table 2.1). After two weeks, reduced velocity increased evenness slightly in all treatment combinations, except for when combined with nutrient enrichment and sediment addition, where evenness was lower at reduced velocity (Fig. S2.5). After three weeks, these patterns had been largely reversed, with evenness decreasing at reduced velocity in two of the four treatment combinations involving sediment addition and nutrient enrichment.

2.4.4. Community compositional changes

The multivariate PERMANOVA results (Table S2.1) showed that total fungal community composition (including rare taxa) changed due to nutrient enrichment ($F_{1,48}$ =8.76, p=0.001) and flow velocity reduction ($F_{1,48}$ =2.45, p=0.02). Further, both PERMANOVA and PCoA results suggested that nutrient enrichment and flow velocity reduction effects changed with time (Table S2.1, Fig. 2.4). Total bacterial community composition was unaffected by all four experimental factors or their interactions. Similar community-level response patterns were also found for bacterial and fungal community composition in the multivariate results of the MANOVAs based only on the abundant taxa (Table 2.2).

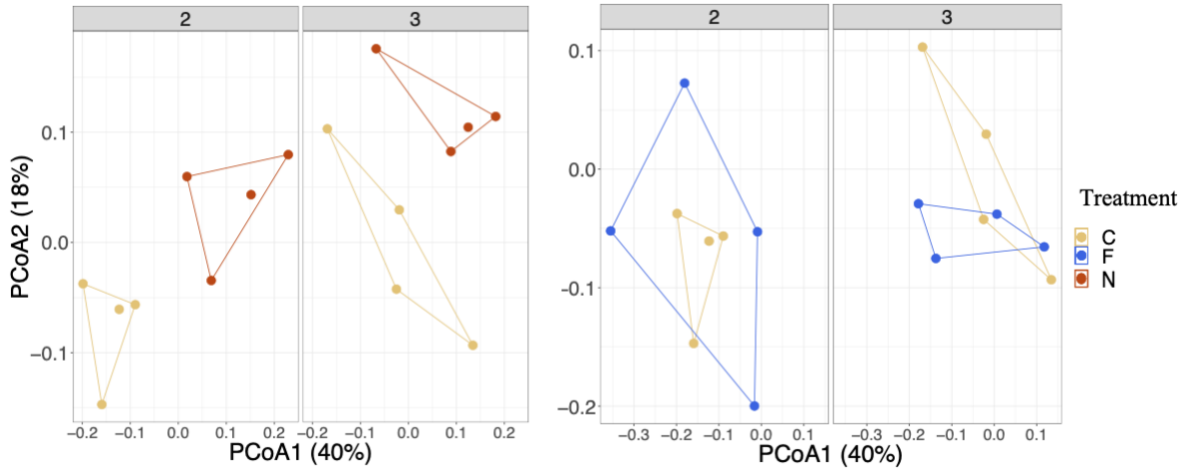


Figure 2.4: PCoA graphical representation of the fungal communities for the nutrient and flow velocity treatments after 2 weeks and 3 weeks of stressor exposure. C is the control treatment, N the nutrient enrichment treatment (without any other stressors added) and F the flow reduction tent (without any other stressors added).

Table 2.2: Summary (p-values and effect sizes) of multi- and univariate LM results for the abundant taxa, with relative abundance by genera as the response variable. For all manipulated factors, significant main effects are classified directionally as positive (+) or negative (–). P-values in bold font where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.1$.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Bacterial community	0.94	0.19	0.70	0.41	0.85	0.31	0.76	0.14	0.46	0.91	0.58	0.31	0.17	0.19	0.51
Acidovorax	0.42	0.93	0.56	0.41	0.56	0.70	0.16	**0.047** (0.08)	0.09 (0.06)	0.53	0.65	0.099 (0.05)	0.38	0.16	0.08 (0.06)
Actinoplanes	0.89	0.81	**0.04** (0.09) +	0.66	0.62	0.18	0.55	0.67	0.61	0.57	0.54	0.42	0.54	0.47	0.72
Aquabacterium	0.82	0.75	0.86	**0.01** (0.11) +	0.39	0.24	0.68	0.92	0.21	0.18	0.78	0.53	0.09	0.31	0.68
Conexibacter	0.88	0.26	0.73	0.11	0.20	0.62	0.48	0.92	0.07 (0.07)	0.25	0.23	0.54	0.65	0.37	0.32
Gemmobacter	0.84	0.35	0.19	**0.03** (0.09) –	0.82	0.65	0.50	0.14	0.28	0.60	0.08 (0.06)	0.55	0.053 (0.07)	0.10	0.48
Herbaspirillum	0.46	0.48	0.35	0.81	0.70	0.25	0.96	0.64	0.30	0.16	0.47	0.89	0.74	0.31	0.97
Lonsdalea	0.24	0.13	0.59	0.86	0.60	0.65	0.54	0.23	0.26	0.13	0.42	0.72	0.26	0.87	0.87
Novosphingobium	0.22	0.43	0.47	0.97	0.39	0.59	0.96	0.36	0.21	0.27	0.54	0.14	0.25	0.31	0.64
Pantoea	0.79	0.99	0.13	0.99	0.28	0.78	0.62	0.54	0.56	0.35	0.72	**0.01** (0.11)	0.28	0.69	**0.04** (0.08)
Pseudomonas	0.90	0.58	0.39	0.99	0.98	0.62	0.34	0.74	0.74	0.44	0.39	0.63	0.44	0.58	0.13
Rhizobacter	0.95	0.37	0.28	0.40	0.42	0.86	0.28	0.40	0.54	0.16	0.33	**0.02** (0.14)	0.92	0.36	0.38
Rhodoferax	0.40	0.20	0.65	0.18	0.92	0.73	0.80	0.31	0.53	0.08 (0.06)	0.40	0.90	0.39	0.30	0.94
Roseateles	0.10	0.79	0.40	0.43	1.00	**0.04**	0.08	0.96	0.89	0.60	0.67	**0.02**	**0.01**	0.54	0.12

						(0.08)	(0.06)					(0.10)	(0.12)		
Sphaerotilus	0.58	0.24	0.27	0.28	0.53	0.40	0.35	0.75	0.76	0.56	0.90	0.81	0.54	0.15	0.59
Sphingobium	0.89	0.58	0.57	0.34	0.51	0.91	0.71	0.75	0.61	0.48	0.47	0.84	0.25	0.44	0.93
Sphingomonas	0.75	0.48	0.28	0.63	0.70	0.06 (0.07)	0.60	0.80	0.08 (0.06)	0.35	0.09	0.53	0.53	0.41	0.35
Sphingorhabdus	0.79	0.40	0.84	0.12	0.73	0.99	0.28	0.50	0.54	0.37	**0.03** (0.09)	0.08 (0.06)	0.26	0.22	0.90
Fungal community	<**0.001** (0.75)	0.74	0.10	**0.04** (0.34)	0.33	0.82	0.31	0.15	0.53	0.14	0.93	1.00	0.61	0.79	0.38
Amphisphaeria	0.62	0.31	0.20	0.94	0.99	0.30	0.052 (0.08)	0.81	0.34	0.45	0.99	0.86	0.72	0.46	0.52
Ascocoryne	0.15	0.94	0.51	0.18	0.40	0.51	0.80	0.68	0.84	0.06 (0.07)	0.70	0.98	0.71	0.99	0.37
Bartalinia	<**0.001** (0.25) –	0.89	0.16	**0.02** (0.11) –	0.52	0.68	0.39	**0.03** (0.09)	0.87	0.91	0.84	0.75	0.93	0.25	0.68
Curvularia	**0.005** (0.15) –	0.25	0.29	0.50	**0.04** (0.08)	0.75	0.88	**0.03** (0.09)	0.30	0.77	0.26	0.63	0.24	0.64	0.65
Dothidea	**0.04** (0.09) –	0.14	0.24	0.50	0.07 (0.06)	0.54	0.50	0.79	0.71	0.21	0.92	0.58	0.74	0.72	0.80
Goniopila	0.22	0.51	**0.03** (0.09) –	0.14	0.68	0.65	0.22	0.78	**0.04** (0.08)	0.71	0.29	0.88	0.58	0.64	0.09
Lunulospora	<**0.001** (0.26) +	0.35	0.52	**0.04** (0.08) –	0.38	0.71	0.64	0.08 (0.06)	0.80	0.30	0.76	0.72	0.06 (0.07)	0.72	0.86
Microdochium	0.06 (0.07)	0.61	**0.03** (0.09) –	0.07 (0.07)	0.70	0.32	0.40	0.25	0.88	0.65	0.64	0.92	0.43	0.41	0.75
Pyrenochaeta	<**0.001** (0.29) +	0.55	0.48	0.17	0.32	0.33	0.83	0.60	0.25	**0.04** (0.09)	0.88	0.79	0.17	0.36	0.70

The univariate results of the MANOVAs indicated that most of the 17 abundant bacterial genera remained unaffected by the stressors, whereas most of the nine abundant fungal genera responded to either flow velocity reduction or nutrient addition (Table 2.2). Indeed, 55.5 % of the abundant fungal genera showed significant main effects for nutrients (*Lunulospora* and *Pyrenochaeta* positive, *Bartalinia*, *Curvularia* and *Dothidea* negative), 22.2 % for flow velocity reduction (*Bartalinia* and *Microdochium*, both negative), and 22.2 % for sampling date (*Bartalinia* and *Lunulospora*, both becoming less prevalent after 3 weeks), whereas all nine genera were unaffected by sediment addition. Among the abundant bacterial genera, only *Actinoplanes* showed a main effect of flow velocity reduction (positive), and 11.7 % of the genera displayed an effect of sampling date (*Aquabacterium* becoming more prevalent after 3 weeks, and *Gemmobacter* less prevalent).

Stressor interactions, including time variations, occurred more frequently in fungal rather than bacterial taxa (44.4 % and 29.4 % respectively) (see Supplementary Material for genus-specific bar graphs and interaction plots). Most bacterial interactive patterns changed through time; except for *Sphingorhabdus* in which nutrient addition moderated the negative effect of sediment and flow velocity reduction (Fig. S2.6.8). For *Acidovorax*, a positive effect of nutrient enrichment after 2 weeks of exposure became negative after 3 weeks (nutrients × time; Fig. S2.6.11). After 2 weeks, *Roseateles* and *Rhizobacter* increased in prevalence when sediment alone was added but decreased when nutrients were also added; after 3 weeks these patterns were reversed, and in both weeks the exact opposite patterns were observed for *Pantoea* – (sediment × nutrients × time; Fig. S6.16-21). Further, after 2 weeks *Roseateles* decreased under flow velocity reduction alone but increased when nutrients were added too, and these patterns were reversed after 3 weeks (flow velocity × nutrients × time; Fig. S2.6.14-15). Finally, *Pantoea* showed a weak, complex 4-way interaction among all manipulated factors (Fig. S2.6.10), which overlaid the stronger 3-way interaction described above.

Regarding interactive effects on abundant fungal genera, *Curvularia* increased in prevalence when sediment alone was added, whereas this genus decreased when nutrients were also added (sediment × nutrients; Fig. S2.6.12). The remaining interactions all involved temporal changes. *Goniopila* responded positively to sediment addition after 2 weeks but negatively after 3 weeks (sediment × time; Fig. S2.6.7), and *Pyrenochaeta* showed the same temporal change for reduced flow velocity (flow velocity × time; Fig. S2.6.9). Finally, the negative effect of nutrient

enrichment was stronger after 2 weeks for *Bartalinia* and after 3 weeks for *Curvularia* (nutrients × time; Figs S2.6.5, S2.6.6).

2.4.5. Persistence of terrestrial microbes after submersion

Most of the terrestrial bacterial and fungal taxa were maintained in the experimental treatments after submersion and exposure to the stressors; however, their relative abundances were drastically altered, especially for fungi (Fig. 2.3, S2.2-S2.3). Terrestrial bacterial and fungal communities were composed of 280 and 56 genera, respectively. All these bacterial genera were detected in at least one treatment replicate on both sampling dates. For fungi, 88 % of the terrestrial community was found in at least one treatment replicate after two weeks of incubation and 94 % after three weeks.

The terrestrial bacterial community was predominantly composed of *Actinoplanes*, *Sphingobium*, *Phenylobacterium*, *Novosphingobium* and *Rhizobacter* (Fig. S2.2), and these five genera also showed high mean relative abundances across treatments (except for *Phenylobacterium*). The terrestrial fungal community was dominated by *Knufia*, *Macrophomina*, *Corynespora*, *Wiesneriomyces* and *Macrodochium* (Fig. S2.3). Although these genera were still detected post submersion and treatment exposure, their relative abundances were much lower in all treatments.

2.5. Discussion

Predicting the composition of microbial communities following exposure to stressors and the functional impacts of any changes are important objectives of microbial ecology. Our experiment used culture-independent techniques, combined with a technologically advanced stream mesocosm system, to investigate the effect of multiple stressors on microbial dynamics involved with leaf litter decomposition in a semi-natural, multi-trophic context and along a short temporal gradient. Under nutrient enrichment, fungal community composition shifted due to changes in relative abundances of certain genera. These changes differed from those found when flow velocity was reduced. Fungal community changes were paralleled by acceleration of decomposition under nutrient enrichment, and deceleration under flow velocity reduction. Compositional changes were characterised by a steep increase in the relative abundances of pre-existing fungal genera such as *Ascocoryne* and *Bartalinia*, which had already colonised the litter in low abundances at the time of leaf senescence. The changes in the relative abundances

rather than identity of the communities suggest that in the early stages of organic matter decomposition, instead of eliminating certain genera or encouraging new ones, our stressors induced a shift in genus prevalence patterns.

Summed across our diversity and common taxa response variables, significant interactions among nutrients, sediment and/or flow velocity (12 in total) were more common than the main effects of these three stressors (10 in total), highlighting the importance of using a full-factorial design in our experiment. This pattern was especially evident for the bacterial response variables, which were affected predominantly via stressor interactions rather than via main effects (9 versus 2), as discussed further below.

2.5.1. The three stressors compared

Our first and second hypotheses – higher decomposition with enriched nutrients and lower with flow velocity reduction – were supported by the observed decomposition rates in these treatments. Our finding for nutrient enrichment is in accordance with previous studies where enrichment increased the metabolic rate of the microbial communities (Manning et al., 2018; Piggott et al., 2015a). Indeed, microbes can easily assimilate resources directly available in the water column at a lower energetic cost (Fernandes et al., 2014; Gulis et al., 2017; Lin and Webster, 2014; Webster et al., 2009). These resources can then be re-mobilised to increase the activity of enzymes involved in the degradation of complex carbohydrates and phenolic compounds (Carreiro et al., 2000). At reduced flow velocity, an increase in the boundary layer thickness surrounding the microbiome is a likely driver of slower decomposition rates (Bruder et al., 2016a; Mustonen et al., 2016; Piggott et al., 2015a). Thus, a thicker boundary layer has been associated with impeding the exchange of resources, such as nutrients and oxygen, between microbiome and water column, leading to reduced microbial activity (Barker Jømgensen and Des Marais, 1990; Lemly, 1982; Stevens and Kurd, 1997). It is unlikely that decreased physical abrasion was responsible for our observations as our normal flow velocity treatments were not fast enough to cause obvious loss of leaf material (N.P.D. Juvigny-Khenafou, personal observation) and decomposition did not advance to the later stages where leaves become easily friable (mass loss did not exceed 50 % of the original mass).

The changes in decomposition rates observed in the flow velocity reduction and nutrient enrichment treatments were paralleled by diversity changes in the fungal community. We had

hypothesised that changes in the identity of microbial genera and in their diversity would occur, but this was supported only for one diversity metric for the fungal community, where taxon richness increased when nutrients where added. Instead, the remaining stressor effects were mainly changes in relative abundances, creating distinct groups of dominant fungal genera in response to the flow velocity and nutrient manipulations. Species in microbial communities have a degree of functional redundancy (Bell et al., 2005; Gessner et al., 2010), and this redundancy may have allowed maintaining most genera across most of our experimental treatments. Moreover, our stream-connected system had a continual input of microbes which could have recolonised the mesocosm substrata, buffering losses of the original genera resulting from the different treatments. Overall, our results indicate that – in a 42-day experiment in a mesocosm system fed by a montane stream – abundance dominance patterns rather than richness drove microbial community shifts in response to stressors.

Our third hypothesis – decreased decomposition with increased sedimentation – was not supported: the sediment treatment affected neither decomposition rates nor microbial community structure. This result is largely divergent from previous studies which usually found a strong effect of sedimentation on decomposition rates and bacterial assemblages whether positive or negative (Bruder et al., 2016a; Matthaei et al., 2010; Romero et al., 2019b). We suspect the sedimentation level applied in our experiment was not high enough to create a strong barrier with the external environment for all the leaves in the packs (see Section 2.3). This idea is further supported by the lack of sediment main effects on microbial community alpha diversity and abundant genera (although several interactive stressor effects involving sediment occurred, see next section).

2.5.2. Stressor main effects and interactions across time

Stressor interactions were more common than stressor main effects, especially for the bacterial community. Most of the significant stressor interactions involved nutrients × sediment and nutrients × flow, often associated with changes from 2 to 3 weeks of stressor exposure. The observed interactive patterns largely supported our fourth hypothesis, that positive nutrient effects on decomposition rates and microbial diversity would be counteracted by sedimentation and flow velocity reduction (although no interactive effects were observed for decomposition rates). A nutrients × sediment × time interaction affected bacterial diversity, whilst a nutrients × flow × time interaction affected fungal richness and a 4-way interaction of all manipulated factors affected both bacterial and fungal evenness. As predicted, when combined with nutrient

enrichment, sediment addition and flow velocity reduction had the opposite effects to that of only nutrient enrichment. We suspect that both sediment addition and flow velocity reduction create physical barriers in the boundary layer, which limits resource exchange between microbiome and water column and prevents a nutrient-enrichment effect. However, alpha diversity patterns were not matched by the total microbial genera turnover analysis where no interactions were found, suggesting a weak influence of community identity on the overall community.

Taxon-specific responses of the abundant microbial genera also followed similar patterns, with most significant interactions involving either nutrients × sediment or nutrients × flow, with a mitigating effect of nutrient enrichment counteracting negative effects of sedimentation or flow velocity reduction in several cases (except for *Curvularia*). Notably this mitigating effect was often inconsistent through time, either lagging (only observed after 3 weeks) or fading (ending after 2 weeks). Previous experiments in terrestrial microbiomes have found that nutrient enrichment accelerated microbial succession (Knelman et al., 2014); thus, the temporal taxon-specific interactive patterns observed in our study could be the result of changes in taxon-specific successional dynamics.

Similar to their interactive effects, stressor main effects on the microbial response variables often changed with time in our study, with several response patterns being reversed from 2 weeks to 3 weeks of stressor exposure. Such temporal effect reversals occurred for abundant individual bacterial (*Acidovorax*) and fungal taxa (*Goniopila, Pyrenochaeta, Bartalinia and Curvularia*). This finding is particularly interesting as temporal patterns are still largely unexplored in microbial community responses to multiple interacting stressors. We further speculate that taken into a community-network context, some biotic interactions among species confer a resistance level to individual microbial taxa, buffering their response to stressors (Tylianakis et al., 2010). However, biotic interactions change through time (Hutchinson et al., 2019), and new interactions can be created whilst others can be lost depending on other species' responses to stressors through time. We suggest that to better understand interactive stressor effects on microbial communities, future efforts should focus on biotic interactions, microbial succession and response thresholds to stressors.

2.5.3. Stochastic versus deterministic effects of stressors on microbial community composition

A previous experiment suggested that stressors, or disturbances, can mediate stochastic community assembly by filtering out unsuitable species (Herren et al., 2016). If stressor levels are strong enough to selectively prevent establishment of certain species, then specific groups of microbes should become associated with the different treatments, and consistent community structures should be found across treatment replicates. The close proximity of the different replicates in our PCoA analysis combined with the selection of specific fungal groups and the overall fungal community structural rearrangements under nutrient enrichment and flow velocity reduction suggests that both stressors can drive succession of the litter fungal community, thus implying deterministic changes. Microbial communities have been hypothesised to have a high functional redundancy because of their high diversity (Bell et al., 2005; Martínez and Canhoto, 2019). However, the lack of obvious selection patterns in most of our stressor combination treatments, combined with high variability between treatment replicates and unchanged decomposition rates, lean towards this functional redundancy hypothesis, indicating a resistance of the overall function of the decomposing litter microbial community to moderate stressors and stressor combinations.

2.5.4. The terrestrial microbial community matters

Our results also suggest that the microbial endosphere and phyllosphere of terrestrial leaves were not replaced during 3 weeks of submergence and stressor exposure. This result is in accordance with Röhl et al. (2017), who also found that a large proportion of the terrestial microbial community persisted in the first three weeks of submerged leaf litter decomposition. Rather, the community structural changes observed in our experiment were largely driven by rearrangements of relative abundances; distinct groups of minor genera present during the terrestrial stage (*Ascocoryne*, *Goniopila*, *Pyrenochaeta*, *Lunulospora*, *Microdochium*, *Amphisphaeria*, *Dothidea*, *Curvularia*, and *Bartalinia*) became dominant after two and three weeks of stressor exposure in stream water and displayed different responses to the stressor treatments. Despite evidence of terrestrial fungi in decomposing stream litter, the role of the initial microbial community has rarely been considered in multiple-stressor studies (Mustonen et al., 2016; Röhl et al., 2017). In our study, a large proportion of the bacterial and fungal genera involved in the variability of the microbial community across treatments were present at all time points of assessment (on Day-7, after 2 weeks and 3 weeks of stressor exposure). We therefore suggest that the original terrestrial microbial community that colonised leaf litter prior to senescence and submersion may be involved in the decomposition process. This

finding is particularly interesting as it would provide a direct link between the terrestrial and aquatic environment. Because most related laboratory experiments involved sterilised leaves and relied on fungal sporulation to assess and/or identify the fungal community, they could not investigate this question (Artigas et al., 2008; Gardeström et al., 2016; Pascoal and Cássio, 2004; Suberkropp, 1998). This difference further highlights the need for more molecular studies on aquatic leaf litter decomposition to be conducted in mesocosms that can realistically simulate stream environments to better understand the mechanisms involved.

2.5.5. Limitations and Conclusions

Our findings suggest that leaf-litter microbial communities have a tolerance level to moderate stressor addition. This point is interesting as our study was conducted in a pristine montane stream, implying that microbial decomposers may have a certain degree of natural resistance to stressor interactions displayed in both their community structure and their activities. Additionally, the absence of interactions among our manipulated stressors observed for leaf decomposition rates matches similar studies (Matthaei et al., 2010; Mustonen et al., 2016; Piggott et al., 2015a).

Our study has several limitations worth being aware of. We did not investigate any biological drivers that may have influenced the leaf litter microbial communities besides the three manipulated stressors, for example detritivorous stream invertebrates. Some invertebrates are known to selectively feed on microbes growing on submerged leaves and could have created varying levels of grazing pressure depending on their own density response to the stressors, thus potentially changing both microbial community composition and decomposition rates (Danger et al., 2016). Further, our experiment dealt only with relatively early stages of leaf decomposition, as can be seen in the moderate mean mass losses (34-37%) during our 4-week incubation period, perhaps due to the use of a tree species with waxy leaves which can prevent fast colonisation by aquatic fungi. Consequently, microbial community replacements patterns might have become stronger had we been able to continue our experiment for several more weeks. Finally, a better characterisation of the fungal community might have been obtained had we combined our 18S approach with ITS primers; this approach might have avoided biases associated with the different primers whilst following the commonly used primers for fungal communities assessments (De Filippis et al., 2017).

One previous study suggested that fungal biomass changes were more important than fungal identity in mediating litter decomposition (Ferreira and Chauvet, 2012), whereas others found relationships with microbial diversity (Costantini and Rossi, 2010; Duarte et al., 2006; Santschi et al., 2018). We did not determine microbial biomass so we cannot directly compare our results to Ferreira & Chauvet (2012), but our results do not support an effect of diversity. Such comparisons need to be made carefully, however, since these studies were conducted at different timescales. Additionally, working with relative rather than absolute microbial abundances may mean that some of the observed patterns in community composition can result from relic DNA that may linger in the aquatic system. Previous experiments have indeed shown that such relic DNA can influence relative abundance patterns of specific microbial taxa, whereas community structure remained unaffected (Gustave et al., 2019).

To conclude, further experiments combining high-throughput sequencing and metabarcoding with metabolic and enzymatic assays should be performed to better understand the microbial communities and their functional responses to multiple stressors in streams. Indeed, modern techniques, such as (meta)genomics, metabolomics, (meta)transcriptomics and (meta)proteomics, can offer unprecedented opportunities to investigate microbial communities' complexity and function *in situ*, greatly enhancing our knowledge beyond what can be acquired from laboratory experiments. When combined with ecosystem function metrics, these techniques have the potential to investigate leaf microbiome responses to stressors from molecules to species in their natural environments.

3. Chapter 3: Sediments and flow velocity impact bacterial community composition and functional profile more than nutrient enrichment

3.1. Abstract

Freshwater ecosystems face many simultaneous pressures due to human activities. Consequently, there has been a rapid loss of freshwater biodiversity and an increase in biomonitoring programs. The objective of this study is to assess the potential of benthic bacterial communities as early indicators of the impacts of multiple stressors associated with urbanisation and agricultural intensification. We conducted a fully crossed 4-factor experiment in 64 flow-through mesocosms fed by a pristine montane stream (21 days of colonisation, 21 days of manipulation) and investigated the effects of nutrient enrichment, flow velocity reduction and sedimentation after 2 and 3 weeks of stressor exposure. We used high-throughput sequencing and metabarcoding techniques (16S rRNA) as well as curated biological databases (METAGENassit, MetaCyc) to identify changes in bacterial relative abundances and predicted metabolic functional profile. Stressors and stressor combinations had pervasive effects on bacterial community composition and predicted functions, and many complex 2-way or 3-way interactions among stressors occurred. Observed changes were largely stable over time and occurred after just 2 weeks of exposure, demonstrating that bacterial communities can be well-suited for early detection of multiple stressors. When combined, changes in bacterial communities and predicted metabolic functions allowed the extrapolation of underlaying stream wide mechanisms operating in the system such as carbon use and bacterial energy production pathways. To conclude, a holistic approach to multiple stressors, which includes basal and higher trophic levels and functional responses, enhances mechanistic understanding of stressor effects, promoting the establishment of more efficient biomonitoring programs.

3.2. Introduction

In many regions of the world, the proportion of freshwater systems subject to multiple stressors outweighs that of pristine systems (Davis et al., 2010; Han et al., 2016; Heathwaite, 2010; Reid et al., 2019). Stressors are largely associated with land-use change, and the Anthropocene has triggered severe disruptions in the natural cycles of resources and freshwater communities (Dudgeon et al., 2006; Reid et al., 2019). For instance, eutrophication has become more frequent as a result of agricultural intensification (Zhao et al., 2006);allochthonous carbon inputs are mediated by riparian land-use changes (Chauvet et al., 2016); and stream communities are becoming simplified due to habitat homogenisation (Petsch et al., 2017). Because of the importance inland waters play in the sustainability of both human and wild communities, it is essential that we understand the consequences of stressor interactions before ecological regime shifts become irreversible (Gordon et al., 2008).

In lotic environments, bacterial communities can be free-living or form conglomerates on bed substrata (biofilms). Bacteria can be autotrophs or heterotrophs, feeding on different carbon sources (Zeglin, 2015). Biofilm and sediment constitute an important food source for higher trophic levels including many invertebrates (Cross et al., 2003; Guilini et al., 2010). Biofilms also provide important functions associated with stream ecosystem health and the maintenance of water quality, such as denitrification, bioremediation and whole-stream respiration (Besemer, 2015; Romaní et al., 2006; von Schiller et al., 2017). Despite their importance, the successional dynamics and function changes of biofilm communities under multiple stressors are still poorly understood, especially in lotic environments (Antwis et al., 2017; Battin et al., 2016).

Bacterial communities have a fast turnover rate and a degree of functional redundancy, thus creating uncertainty in the extent to which microbiota are sensitive, resistant or resilient to multiple stressors (Antwis et al., 2017; Bell et al., 2005). Further, environmental parameters alone do not always explain response to stress as bacterial populations directly or indirectly interact between each other, forming a network of non-random associations between taxa (Barberán et al., 2012; Battin et al., 2016; Lima-Mendez et al., 2015; Widder et al., 2016). If multiple stressors have strong impacts on bacterial community assemblages and/or function, these changes can be regarded as adaptive responses to stress over short time periods. By contrast, if functional redundancy dominates, communities should remain largely static or in

completely random associative patterns (Herren et al., 2016). If the former scenario of strong impacts is correct, benthic bacterial communities could be useful indicators of multiple stressor effects (Romero et al., 2018). Indeed, bacterial communities may sometimes detect stressor effects before they can be observed in higher trophic levels such as invertebrates or fishes. Because of their longer lifespans, unless a catastrophic event occurs, organisms at higher trophic levels may persist in streams longer after their fitness decreases, thus changes in fish or invertebrate community composition may be slower to become evident (He et al., 2019). Equally, because of their basal resource position in the food web, changes in biofilm characteristics (e.g. composition, biomass, function) have the potential to modulate the density of higher trophic levels (Antwis et al., 2017). For instance, Ullah et al. (2018) found in marine systems that the combined stress of acidification and warming reduced the biomass flows from the first trophic level (primary producers and detritus) to the second (herbivores), and from the second to the third (carnivores).

Stressors such as sedimentation, water scarcity and nutrient enrichment, which are often associated with urbanisation and agricultural land uses, are known to have a strong impact on stream microbial communities and their activities (Pascoal and Cássio, 2004; Piggott et al., 2015a; Widder et al., 2014). Both sedimentation and reduced flow velocity limit the exchange of resources between the water column and benthic habitats by creating a physical barrier and increasing boundary layer thickness (Barker Jømgensen and Des Marais, 1990; Stevens and Kurd, 1997), whilst eutrophication favours development of microbes associated with algal blooms (Piggott et al., 2015b) and has been seen to accelerate microbial succession in terrestrial systems (Knelman et al., 2014). However, other experiments on stream benthos and sediments have also reported limited effect of nutrient enrichment on bacterial communities (Bowen et al., 2011; Salis et al., 2017). Sedimentation also changes the physical structure of the substrata on which bacteria grow, influencing bacterial attachment and development (Allan, 2004; Romero et al., 2019b; Salis et al., 2017). Overall, stressors influencing the metabolic rate of microbial communities then modulate successional dynamics. To date, studies of bacterial community composition under different environmental conditions often involve field observations, which may lack the ability to reveal mechanistic understanding, or laboratory assays with insufficient complexity of the microbial community and abiotic parameters compared with that of the natural environment (Romero et al., 2018; Steen et al., 2019; Wagner et al., 2015). We argue that to better understand the shifts in microbial community composition and function subject to multiple stressors, we must consider various mechanisms including

adaptation, selection, dispersal, dormancy, persistence, co-occurrence, priority effects, legacy effects, and interspecific interactions as part of the experimental design (Bissett et al., 2013). Thus, a capacity to control environmental variables, coupled with the full complexity of microbial communities, is necessary, making field manipulative studies more realistic and relevant to natural conditions.

Here we used flow-through field stream mesocosms to investigate the effects of nutrient enrichment, flow-velocity reduction and fine-sediment deposition on benthic bacterial communities. Our overall aims were to investigate the community assembly processes under multiple stressors, and to examine how these processes shape the functional profile of the assemblages (Figure 3.1). We tested five specific hypotheses: 1. Bacterial diversity will have a certain degree of resistance to nutrient enrichment but successional dynamics will be accelerated, causing changes in dominance patterns (Bowen et al., 2011; Knelman et al., 2014); 2. Flow-velocity reduction and added fine sediment will create strong community assemblage shifts, by changing the physical environment and influencing dispersal and settlement of biofilm (Besemer et al., 2009; Salis et al., 2017); 3. Stressor-induced changes in benthic bacterial communities will drive shifts in their metabolic functional profile which are linked to major stream ecosystem processes such as carbon and nutrient cycles (Romero et al., 2019a, 2019b); 4. The three stressors will interact when affecting bacterial communities, resulting in a range of complex responses (Salis et al., 2017); 5. There are non-random interactions between groups of dominant bacterial genera (Lima-Mendez et al., 2015).

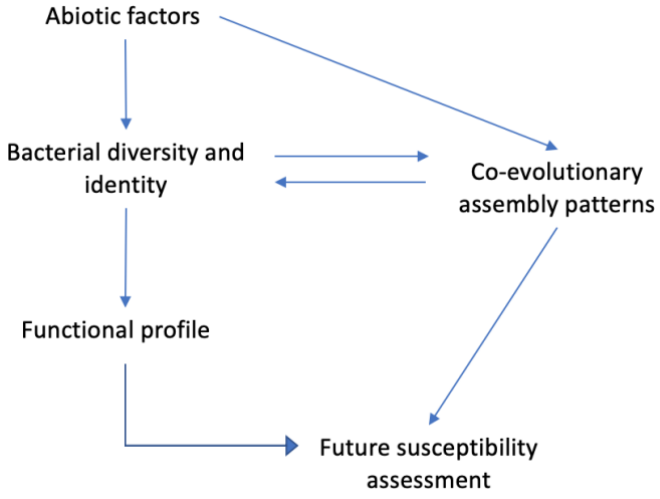


Figure 3.1: Framework of the study objectives. Abiotic parameters interact with each other's to modify the bacterial community network. Taxa which have evolved in non-random association are expected to respond to the same stressors which may accelerate or decelerate

the evolutionary trajectory adopted by the prior stress community. This results in shifts in the community functional profile and stream wide processed (nutrient cycling, carbon cycling and others). By combining the functional profile of the community its assembly organisation, the importance of various taxa in controlling the community functionality and organisation can be evaluated and further susceptibility of the system extrapolated.

3.3. Methods

3.3.1. Experimental system

See Chapters 2.3.1, 2.3.2.

3.3.2. Experimental design

We manipulated deposited fine sediment on the bed surface (ambient vs. added), dissolved nutrient concentrations (ambient vs. enriched) and flow velocity (fast vs. reduced) in a full-factorial design with two sampling dates (Days 13 and 20). A detailed description of the study design and the experimental manipulations can be found in Chapter 2.3.2 and 2.3.3.

3.3.3. Benthic biofilm sampling

On Days 13 and 20, benthic biofilm samples from ceramic tiles (including the associated fine surface sediment) were collected carefully, placed into individual sterile 50-mL centrifuge tubes, and kept on ice before being frozen at -20°C within 2 hrs of collection. When sampling tiles, we first siphoned the top surface of each tile (including any fine sediment present) using 10 mL sterile syringes, and then collected the entire tile. All samples were sent to Sangon Biotech Co., Ltd., Shanghai, China for DNA extraction.

3.3.4. DNA extraction, PCR amplification and sequencing

For each mesocosm, one tile with associated fine surface-sediment was used for the DNA extraction. To extract the microbial DNA, tiles were first scraped with a sterile blade and washed with 1 x phosphate buffered saline solution (PBS) into the centrifuge tubes containing the sediment. Then 2 g of 0.5 - 1 mm sterile glass beads were added to the biofilm and sediment slurry. Centrifuge tubes were vortexed for 3 min at 2500 rpm and homogenised for 5 min before being centrifuged at 12000 g for 2 min. The precipitate was then collected for DNA extraction. All samples were later processed by next-generation sequencing (Illumina MiSeqPE250) using the PCR primer pairs Nobar 341F/Nobar 805R (J. Zhang et al., 2019), targeting the V3-V4 region of bacterial 16s rRNA. Both forward and reverse primers were tagged with adapter

sequences, pad and linker regions and a unique barcode on the forward primer to permit the multiplexing of samples. All primers were provided by Sangon Biotech Co., Ltd., Shanghai, China.

A nested PCR was then implemented. Details of the PCR mixtures, cycles and library-building can be found in Juvigny-Khenafou et al. (2019). Sequences were processed and analysed using QIIME 1.8.0 based on sequence length, quality, primer and tag. The forward and reverse reads were joined with an overlap length of 150 bp. Following removal of the primer, all reads having a tail quality score below 20 (with a 10 bp window), containing ambiguous characters and less than 200 bp were removed. Chimeras were identified using the UCHIME software (Edgar et al., 2011). The filtered reads were then clustered into OTUs (Operational Taxonomic Units) with USEARCH using a 97% similarity (Edgar, 2010) and OTUs found in only one read across the dataset were discarded. The average length of the remaining filtered sequences was 416 bp. The taxonomic assignment of OTUs was performed against the Silva database with the Ribosomal Database Project classifier and a minimal confidence score of 0.8 (Gustave et al., 2019; Purahong et al., 2016; Xiao et al., 2018). We did not rarefy the samples but instead used coverage estimators to ensure their comparability prior to downstream analyses (Chao and Jost, 2012; Shimadzu, 2018). Raw prokaryotic data were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA560484.

3.3.5. Data analysis

All statistical analyses were performed using R (version 3.5.2, R Core Team). The multivariate analyses detailed below were computed with the *vegan* package (Oksanen, 2015). We used a linear model with a significance level and effect sizes following Chapter 2.3.6.

We first generated class-level relative abundance plots for overview purposes and then statistically analysed our dataset at the order level because a previous experiment in a similar mesocosm system revealed that taxonomic resolution below order did not increase detectability of stressor interactions affecting bacterial communities (Salis et al., 2017). Bacterial alpha diversity, richness, Shannon index and Pielou's evenness were computed and analysed with the linear model detailed above.

To investigate the total community response (including rare taxa) to the stressors, a PERMANOVA (Bray-Curtis coefficient and 999 permutations) was performed, and a permutational analysis of multivariate dispersions (PERMDISP; Anderson, 2006) conducted to assess homogeneity. Principal coordinate analysis (PCoA) plots were then made to allow visual representation of significant PERMANOVA outputs. PCoA plots were based on Hellinger-transformed data to reduce the weights for genera with low relative abundance counts and to allow subsequent Euclidian representation of data based on Euclidian distances (Legendre and Gallagher, 2001). A MANOVA analysis was then performed on the abundant taxa (>1% of the total reads, Baltar et al., 2015) to investigate community and taxon-specific responses. Some Spartobacteria taxa could not be assigned to an order and were thus kept as *Spartobacteria_uncl* in this analysis. To further identify taxa of particular interest, a similarity percentage analysis (SIMPER) was performed with a 70% cumulative dissimilartity cut-off (Tolkkinen et al., 2015b). This allowed us to identify the taxa that accounted for most of the differences between the treatments and control mesocosms.

We then built a co-occurrence network between the abundant taxa across all experimental treatments following Widder et al. (2014), to identify highly connected taxa. This network analysis provides insights about the potential biotic interactions among the abundant taxa and allows inferences to be made about the stability of a system (Tylianakis et al., 2010). Briefly, the abundant taxon community matrix was Hellinger-transformed and a pairwise Spearman correlation matrix generated. The significance level was adjusted for false discovery rates following Benjamini & Hochberg (1995), and correlations were regarded as robust if the coefficient ρ_S was > |0.6| and significant at $P < 0.01$. Our sample set covered a wide range of environmental conditions, thus giving us sufficient variability in taxon abundances to resolve co-occurrence patterns (Barberán et al., 2012).

Finally, changes in the functional community profile were assessed by uploading the community matrix to the METAGENassist web server (Arndt et al., 2012). The METAGEN database enables the automated taxonomic-to-phenotypic mapping of the genomic reads. The database of phenotypic information covers nearly 20 functional categories such as oxygen requirements, energy sources and metabolic pathways. For the purpose of our study, we only examined the 'metabolic pathways' functional category to evaluate biosynthesis and assimilation strategies. We then performed a MANOVA analysis with the same structure as above on the abundant predicted functions (>1% of the total reads). Identified abundant

predicted functions were cross-referenced against the MetaCyc database, an online highly curated reference source for metabolic data, to get deeper insight on their functioning (Karp, 2002).

3.4. Results

3.4.1. Class-level microbial community composition

The dataset had a total of 5,105,737 raw reads, out of which 4,761,936 were assigned to 23,952 bacterial OTUs after dereplication, quality filtering and sorting. The Coverage estimator showed that similar degrees of completeness were achieved (Coverage range 0.98 ± 0.001 [SD]) with sufficient sampling depth (16S: 72,507 ± 16,823 [SD]) in each sample.

The community was dominated by Proteobacteria (68.3% of all reads), amongst which Alphaproteobacteria (44.9% after 2 weeks, 43.6% after 3 weeks), Betaproteobacteria (4.5%, 4.6%) and Gammaproteobacteria (19.2%, 19.6%) were the most abundant taxa. At the class level, bacterial communities showed clear rearrangement patterns in response to the experimental treatments, and these changes were largely stable through time (Fig. 3.2). Response patterns at the order level are presented in the section on *Community compositional changes*.

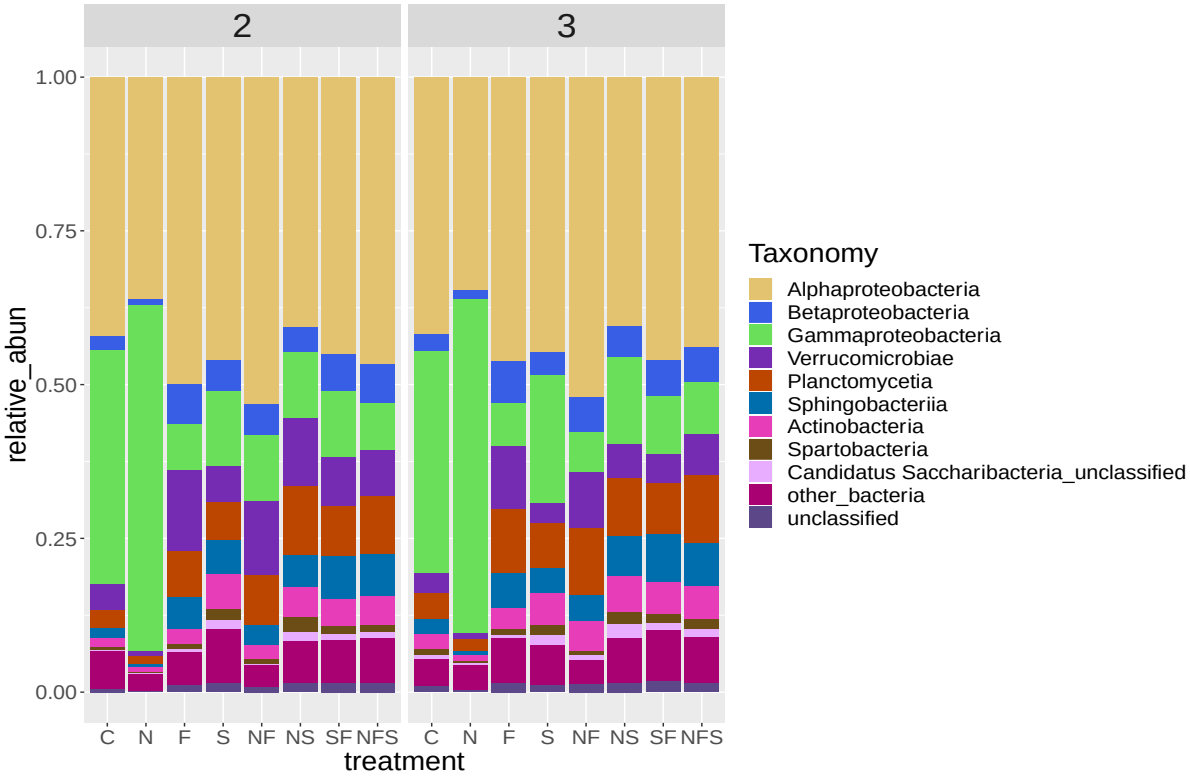


Figure 3.2: Mean relative abundances of bacterial classes across the different treatments after 2 weeks (left) and 3 weeks (right) of exposure to the stressors; C: control, N: nutrients, F: flow velocity, S: sediment, NF: nutrient + flow velocity, NS: nutrient + sediment, SF: sediment + flow velocity, NFS: all three stressors combined.

3.4.2. Stressor effects on alpha diversity metrics

Added sediment and flow-velocity reduction increased bacterial order richness, diversity and evenness, whereas nutrient enrichment had no main effect on these metrics (Table 3.1). Further, interactions between sediment x nutrients and sediment x flow-velocity reduction affected two of the three metrics. Shannon diversity and Pielou's evenness both increased more strongly with added sediment in nutrient-enriched than in non-enriched mesocosms (Fig. S3.1, S3.2), and both metrics also increased more strongly with added sediment in mesocosms with fast flow than in those with reduced flow velocity (Fig. S3.3, S3.4). Only one effect of time was observed, an increase in order richness from week 2 to week 3.

Table 3.1: Summary (p-values and effect sizes) of linear model results comparing bacterial alpha diversity metrics in response to the treatment combinations; nutrient enrichment, sediment addition, flow velocity reduction and time of exposure. For the manipulated factors, main effects are classified directionally as positive (+) or negative (–). P-values are in bold where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.1$.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Richness	0.26	**<0.001** (0.62) +	**0.04** (0.08) +	**0.02** (0.10) +	0.54	0.78	0.13	0.81	0.17	0.85	0.30	0.52	0.58	0.78	0.17
Shannon index	0.06 (0.07)	**<0.001** (0.56) +	**<0.001** (0.31) +	0.25	**0.02** (0.11)	0.53	**<0.001** (0.29)	0.83	0.43	0.81	0.12	0.53	0.80	0.70	0.54
Pielou's evenness	0.053 (0.07)	**<0.001** (0.52) +	**<0.001** (0.33) +	0.39	**0.01** (0.12)	0.48	**<0.001** (0.32)	0.80	0.53	0.79	0.12	0.57	0.87	0.70	0.66

3.4.3. Community compositional changes

The PERMANOVA on the whole bacterial community (including rare taxa) revealed similar responses to the stressors as the diversity metrics, with sediment and flow-velocity reduction but not nutrients having significant stressor main effects, and two-way interactions occurring for sediment x nutrients and sediment x flow-velocity reduction (Table 3.2). These results were largely matched by the multivariate findings of the MANOVA on the abundant-taxon community, except that here nutrient enrichment did have a significant main effect, and a two-way interaction was identified for nutrients x flow velocity. Both communities changed across the two sampling dates, and the abundant-taxon community also showed a flow velocity x time interaction.

The PCoA plots (Fig. 3.3) displayed three main community clusters (i.e. control and nutrient-enriched mesocosms; ii. sediment, nutrients + sediment, sediment + flow velocity reduction, and the three stressors combined; iii. flow velocity reduction and nutrients + flow velocity reduction), with relatively minor changes within these clusters from week 2 to week 3.

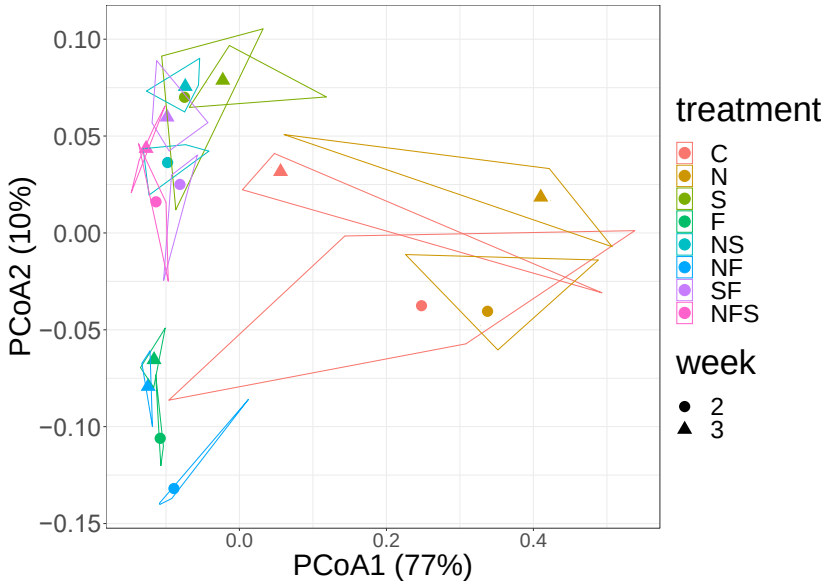


Figure 3.3: PCoA of the different treatments. The points represent the centroids and the polygons are the edges made by the different treatment replicates. C: control, N: nutrient, F: flow velocity, S: sediment, NF: nutrient + flow velocity, NS: nutrient + sediment, SF: sediment + flow velocity, NFS: all three stressors combined.

The univariate results of the MANOVAs (Table 3.2) indicated that all 13 abundant bacterial taxa were affected by at least one of the stressors as a main effect. Thus, 15.4% of the abundant

taxa showed significant main effects for nutrients (*Actinomycetales* and *Sphingobacteriales*, both negative), 76.9% for sediment addition (positive: *Acidimicrobiales*, *Actinomycetales*, *Burkholderiales*, *Planctomycetales*, *Rhizobiales*, *Spartobacteria_uncl*, *Sphingobacteriales*; negative: *Pseudomonadales*, *Sphingomonadales*), 84.6% for flow velocity reduction (positive: *Acidimicrobiales*, *Burkholderiales*, *Planctomycetales*, *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Sphingobacteriales*, *Verucomicrobiales*, *Xanthomonadales*; negative: *Pseudomonadales*, *Sphingomonadales*), and 23.1% for the duration of the experiment (positive: *Acidimicrobiales*, *Rhizobiales*; negative: *Verrucomicrobiales*). Some of these factor main effects for sediment or flow velocity were overridden by stronger interactions of these two stressors, as detailed below. Stressor main effects remained largely stable from week 2 to week 3, except that *Acidimicrobiales* increased more markedly after 3 weeks when flow velocity was reduced (Fig. S3.5), whereas *Actinomycetales* increased more after 2 weeks when sediment was added (Fig. S3.6).

Interactions among the stressors affected all but one (*Rhizobiales*) of the 13 abundant taxa. Nutrients x sediment interactions occurred in seven taxa (53.8%). Nutrients x flow velocity interactions occurred in two taxa (15.4%). Sediment x flow velocity interactions occurred in 12 taxa (92.3%). Three-way interactions among all stressors occurred for four taxa (30.7%). All were relatively weak and did not override any lower-order interactions.

Table 3.2: Results (p-values and partial-η^2 effect sizes) of the PERMANOVA on the whole bacterial community including rare taxa (p-value and F-value) and the MANOVA (multivariate and univariate results) on the 13 abundant bacterial taxa against the different treatment combinations nutrient enrichment, sediment addition, flow velocity reduction and time of exposure. When combined, the abundant taxa accounted for 86.8% of all reads.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Total community (perMANOVA)	0.29 F=1.12	**<0.001** F=32.48	**<0.001** F=66.60	**<0.001** F=2.25	**0.008** F=6.89	0.24 F=1.26	**<0.001** F=34.19	0.79 F=0.21	0.24 F=1.38	0.74 F=0.24	0.06 F=3.38	0.84 F=0.16	0.73 F=0.26	0.51 F=0.50	0.47 F=0.65
Abundant taxa (MANOVA, multivariate results)	**0.003** (0.53)	**<0.001** (0.89)	**<0.001** (0.85)	**<0.001** (0.73)	**0.005** (0.51)	**<0.001** (0.63)	**<0.001** (0.76)	0.50	0.07 (0.40)	**0.01** (0.48)	0.31	0.92	0.07 (0.40)	0.67	0.13
Acidimicrobiales	0.11	**<0.001** (0.52) **+**	**<0.001** (0.36) **+**	**<0.001** (0.23) **+**	0.40	**0.02** (0.11)	**<0.001** (0.39)	0.07 (0.06)	0.28	**0.02** (0.10)	**0.03** (0.09)	0.99	0.22	0.37	0.058 (0.07)
Actinomycetales	**0.04** (0.09) **–**	**<0.001** (0.74) **+**	0.33	0.29	0.99	0.62	**0.02** (0.10)	0.57	**0.03** (0.09)	0.61	0.74	0.94	0.36	0.91	0.15
Burkholderiales	0.25	**0.03** (0.10) **+**	**<0.001** (0.51) **+**	0.47	0.45	0.76	**0.02** (0.11)	0.64	0.18	0.68	0.93	0.95	0.47	0.98	0.20
Planctomycetales	0.17	**<0.001** (0.25) **+**	**<0.001** (0.34) **+**	0.14	**0.02** (0.11)	0.67	**<0.001** (0.24)	0.66	0.32	0.30	0.16	0.89	0.40	0.87	0.53
Pseudomonadales	0.23	**<0.001** (0.47) **–**	**<0.001** (0.63) **–**	0.91	**0.009** (0.13)	0.16	**<0.001** (0.50)	0.62	0.39	0.69	**0.04** (0.08)	1.00	0.87	0.52	0.60
Rhizobiales	0.20	**<0.001** (0.47) **+**	**<0.001** (0.23) **+**	**0.04** (0.08) **+**	0.31	0.23	0.14	0.89	0.92	0.86	0.15	0.78	0.95	0.63	0.20
Rhodobacterales	0.87	0.37	**<0.001** (0.68)	0.07 (0.06)	0.15	**0.03** (0.09)	**<0.001** (0.49)	0.71	0.36	0.66	**0.03** (0.09)	0.68	0.95	0.57	0.97

			+												
Rhodospirillales	0.57	0.27	<**0.001** (0.67) +	0.35	0.10	0.17	<**0.001** (0.56)	0.63	0.39	0.29	0.09 (0.06)	0.90	0.92	0.69	0.34
Spartobacteria uncl	0.71	<**0.001** (0.55) +	0.37	0.23	**0.01** (0.13)	0.79	**0.002** (0.18)	0.39	0.67	0.59	0.26	0.59	0.44	0.23	0.70
Sphingobacteriales	**0.03** (0.09) –	<**0.001** (0.75) +	<**0.001** (0.64) +	0.12	**0.001** (0.18)	0.11	**0.005** (0.15)	0.37	0.49	0.50	0.24	0.19	0.29	0.85	0.058 (0.07)
Sphingomonadales	0.35	**0.02** (0.10) –	<**0.001** (0.50) –	0.77	**0.004** (0.16)	0.55	<**0.001** (0.28)	0.62	0.17	0.81	0.08 (0.06)	0.88	0.99	0.66	0.81
Verrucomicrobiales	0.86	0.81	<**0.001** (0.51) +	<**0.001** (0.22) –	**0.002** (0.18)	0.49	<**0.001** (0.49)	0.92	0.24	0.84	0.09 (0.06)	0.76	0.39	0.09 (0.06)	0.20
Xanthomonadales	0.32	<**0.001** (0.35) +	<**0.001** (0.56) +	0.15	**0.048** (0.08)	0.17	<**0.001** (0.36)	0.34	0.41	0.08 (0.06)	**0.003** (0.16)	0.61	0.53	0.38	0.86

3.4.4. Correlation between abundant taxa

The 13 abundant taxa were highly connected in our network, with a total of 66 statistically robust correlative links identified (22 negatives, 44 positives; Fig. 3.4). On average, each taxon had 5.07 ± 0.95 (SD) interactions with other taxa in the network. *Pseudomonadales* had the highest number of links (7 correlations with other taxa). Further, two clusters can be identified with the phylogenetically related Proteobacteria predominantly having intraspecific correlations, except for *Rhizobiales* which did not share any link with other Proteobacteria. The other group encompassed the other taxa except *Verrucomicrobiales* primarily associated with Proteobacteria. Additionally, pairwise negative relationships, potentially indicating competition or detrimental associations, only occurred when at least one taxon belonged to the Proteobacteria Phylum.

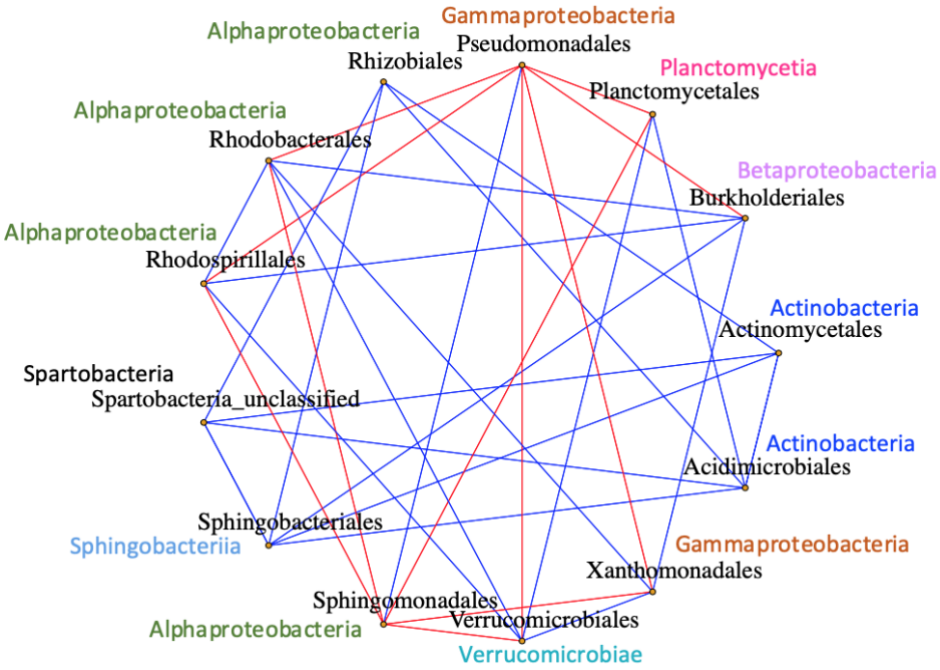


Figure 3.4: Co-occurrence network among the abundant taxa (Genus in black font, and Class in coloured font). Blue lines represent positive relationships in relative abundances whereas red lines indicate negative relationships.

3.4.5. Taxa responsible for most of the deviations from the control community

The SIMPER analysis (Table S3.1) showed that the same groups of 4-5 bacterial taxa accounted for most of the dissimilarity between the control and each stressor treatment. All these taxa were part of the abundant taxon community. *Pseudomonadales* were consistently responsible for the highest proportion of dissimilarity.

3.4.6. Predicted bacterial functions

A total of 15 predicted bacterial metabolic functions were identified in the dataset, with a prevalence of functions associated with ammonia oxidation (19.2%, mean across the whole dataset), dehalogenation (24.5%), sulfate reduction (30.7%) and xylan degradation (8.4%) (Fig. 3.5).

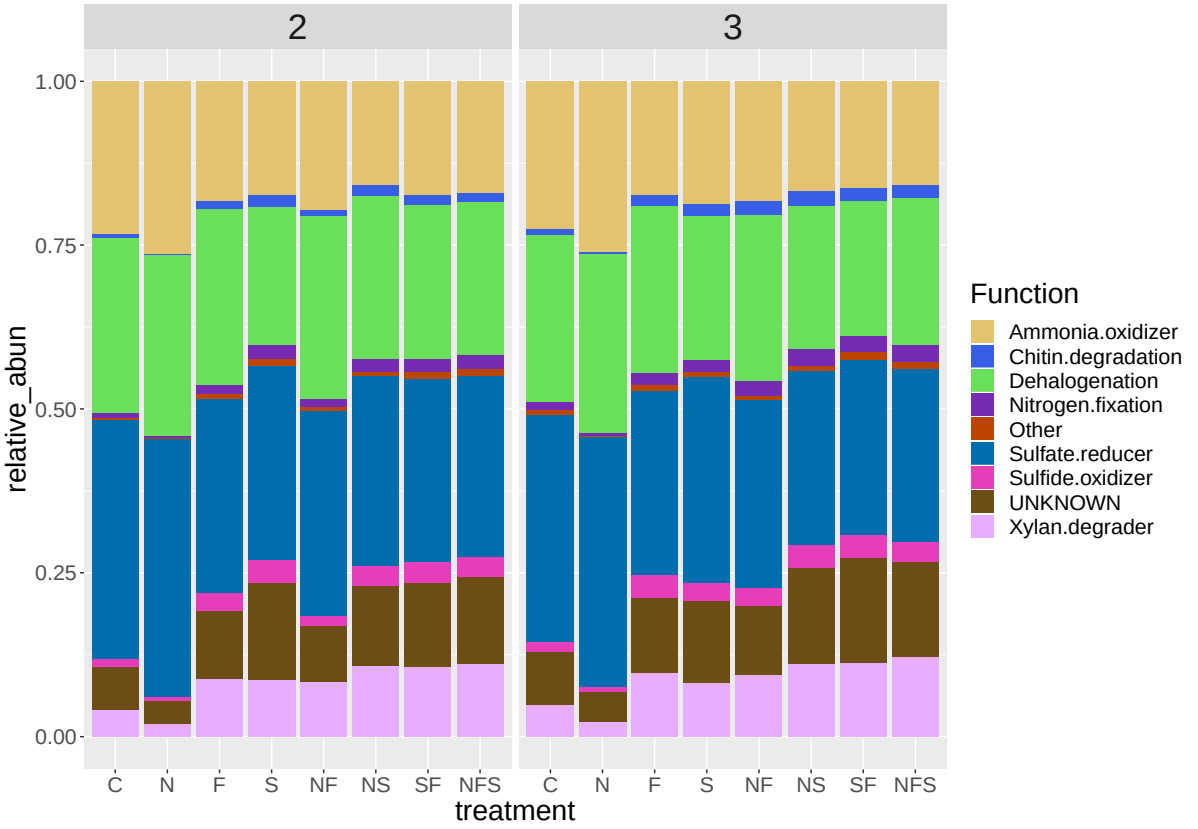


Figure 3.5: Predicted functional profiling of bacterial communities from the METAGENassist prediction tool after two weeks and three weeks of treatment exposure. C: control, N: nutrient, F: flow velocity, S: sediment, NF: nutrient +flow velocity, NS: nutrient + sediment, SF: sediment + flow velocity, NFS: all three stressors combined.

The multivariate results of the MANOVA on the seven abundant predicted functions showed that all three stressor main effects and their 2-way and 3-way interactions influenced the predicted functionality of the bacterial community (Table 3.3). The univariate results indicated that 28.5% of the abundant functions responded to nutrient enrichment (dehalogenizers positively, sulfide oxidizers negatively), 100% to sediment addition (positive: nitrogen fixers, xylan degraders, chitin degraders, sulfide oxidizers; negative: dehalogenizers, ammonia oxidizers, sulfate reducers) and 85.7% to flow-velocity reduction (positive: nitrogen fixers, xylan degraders, chitin degraders, sulfide oxidizers; negative: ammonia oxidizers, sulfate

reducers). Two of these flow velocity main effects were overridden by stronger sediment x flow velocity interactions – see below.

Stressor interactions were common, with four (57.1%) of the seven abundant bacterial functions showing nutrients x sediment, six (85.7%) sediment x flow velocity and three (42.8%) nutrients x sediment x flow velocity interactions. Three-way interactions occurred for three of the seven bacterial functions (42.8%). Finally, two stressor main effects changed with time for sulfide oxidizers (flow velocity x time: positive effect of velocity reduction stronger after 3 weeks than after 2 weeks, Fig. S3.33; sediment x time: positive effect of added sediment weaker after 3 weeks, Fig. S3.34), and so did the shape of the 3-way interaction for three functions (chitin degraders, dehalogenizers, sulfide oxidizers).

Table 3.3: Multivariate and univariate MANOVA results (p-values and partial-η^2 effect sizes) for the METAGENassist six abundant predicted bacterial functions (>1% of the total reads) against the different treatment combinations nutrient enrichment, sediment addition, flow-velocity reduction and time of exposure.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Abundant functions (MANOVA, multivariate results)	<0.001 (0.54)	**<0.001** (0.83)	**<0.001** (0.68)	**<0.001** (0.57)	**0.005** (0.37)	**0.004** (0.38)	**<0.001** (0.64)	0.66	**0.003** (0.38)	0.29	**0.01** (0.33)	0.59	0.24	0.37	0.19
Nitrogen fixation	0.95	**<0.001** (0.66) +	**<0.001** (0.38) +	**<0.001** (0.20) +	0.1	0.1 (0.06)	**<0.001** (0.24)	0.16	0.33	0.18	**0.046** (0.08)	0.36	0.81	0.76	0.07 (0.07)
Dehalogenation	**0.03** (0.17) +	**<0.001** (0.76) –	0.57	**<0.001** (0.26) –	0.47	0.16	0.57	0.35	0.86	0.15	0.98	0.49	0.16	0.84	**0.005** (0.15)
Xylan degrader	0.80	**<0.001** (0.67) +	**<0.001** (0.60) +	0.17	**0.001** (0.20)	0.98	**<0.001** (0.33)	0.76	0.64	0.41	**0.03** (0.09)	0.61	0.91	0.80	0.74
Ammonia oxidizer	0.30	**<0.001** (0.63) –	**<0.001** (0.48) –	0.46	**0.002** (0.18)	0.71	**<0.001** (0.40)	0.90	0.45	0.17	0.09 (0.06)	0.86	0.89	0.42	0.74
Sulfate reducer	0.66	**<0.001** (0.54) –	**<0.001** (0.52) –	0.56	**0.009** (0.13)	0.91	**<0.001** (0.28)	0.39	0.44	0.60	0.11	0.54	0.61	0.91	0.28
Chitin degradation	0.85	**<0.001** (0.64) +	**<0.001** (0.32) +	**<0.001** (0.29) +	0.08 (0.06)	0.35	**<0.001** (0.44)	0.08 (0.06)	0.78	0.06 (0.07)	**0.04** (0.08)	0.87	0.61	0.37	**0.03** (0.09)
Sulfide oxidizer	**0.003** (0.17) –	**<0.001** (0.69) +	**<0.001** (0.46) +	**0.03** (0.09) +	**0.003** (0.17)	0.25	**<0.001** (0.49)	0.26	**0.04** (0.08)	**0.049** (0.08)	0.91	0.46	0.29	0.45	**0.04** (0.08)

3.5. Discussion

Changes in community assembly patterns have traditionally been used as surrogates to identify and understand multiple-stressor effects on ecosystems (Ding et al., 2017; Lau et al., 2015; Piggott et al., 2015b). Notably, bacterial communities and activity have proven to be very useful for evaluating stressor-mediated changes in major biogeochemical processes in streams (Romero et al., 2019b, 2018). In our experiment, benthic bacterial assemblages strongly responded to added fine sediment, flow-velocity reduction and their interaction. Although nutrient enrichment had a lesser influence, it still shifted relative abundance dominance patterns similarly to previous studies (Bowen et al., 2011; Knelman et al., 2014; Vizza et al., 2018). Overall, stressor-related changes in bacterial assemblages drove changes in their functional profile which can be linked to major stream metabolic processes. Our results also indicate that a subset of taxa can be isolated to best describe the stressor response patterns with non-random association patterns between taxa.

3.5.1. The three single stressors compared

Our first hypothesis – bacterial biofilm community structure has a certain resistance to nutrient enrichment but successional rates are accelerated, changing dominance patterns – was largely supported. Nutrient enrichment had no effects on α-diversity metrics or the composition of the entire bacterial community (including rare taxa). However, when only the most abundant taxa were considered, changes in the assemblage structure of these 13 taxa became apparent and two common orders decreased in relative abundance in enriched mesocosms. These results are broadly consistent with those of previous related field surveys and manipulative experiments (Piggott et al., 2015b; Salis et al., 2017; Vizza et al., 2018). To explain these findings, we suggest that most of the bacterial orders in our study system are well-adapted to oligotrophic conditions, their normal environment year-round, and that our nutrient enrichment was not high enough to generate a strong shift in community composition. Nutrient limitation and the degree of homeostasis of bacterial communities are current research topics that have generated rather divergent findings; whether nitrogen or phosphorus is the limiting factor depends on the context and the variable of interest (Danger et al., 2008; Danger and Chauvet, 2013; Vizza et al., 2018). Further, Knelman *et al.* (2014) argued that nutrient enrichment mainly speeds up the succession in microbial communities, and this may have been the case in our experiment because of the slope changes observed in the nutrient combinations. Moreover, the source and

concentration of organic matter are generally stronger determinants of bacterial community composition than eutrophication (Zeglin, 2015). Thus, past experiments have shown that algal-mat development, which often responds positively to nutrient enrichment, can prime the activity of microbial communities by fuelling the detrital pool (Halvorson et al., 2019). In our experiment, this indirect effect of nutrient enrichment may not have been pervasive enough to strongly change bacterial assemblage structure.

Sedimentation and flow-velocity reduction were our most pervasive stressors, as predicted by our second hypothesis, and similar to findings of previous related studies (Piggott et al., 2015b; Romero et al., 2019b; Salis et al., 2017). Both stressors influenced biodiversity, taxon-specific responses and the predicted functional profile, as well as creating distinct clustered communities as seen in our PCoA results. Sedimentation may affect bacterial communities in at least two possible ways. By covering the streambed surface, a physical barrier is created and resource exchanges (e.g. oxygen, nutrients) between the water column and the hyporheic zone are hampered (Hartwig and Borchardt, 2015). Further, the physical characteristics of the bed surface are modified, for example substratum grain size and bed surface roughness are decreased. Together, these changes can favour groups of bacteria well-adapted to the new environmental conditions (Besemer, 2015) and may also influence microbial colonisation patterns. Paralleling findings of a previous related study (Salis et al., 2017), both bacterial diversity and community evenness in our experiment increased in mesocosms containing added fine sediment. This result suggests that bacterial communities in these mesocosms were more diverse because they contained both new taxa preferring fine sediment as well as persisting taxa capable of tolerating sediment-rich conditions.

Flow-velocity reduction increases the boundary-layer thickness above the stream bed, thus decreasing resource exchange rates with the water column (Stevens and Kurd, 1997). On the other hand, a reduction in velocity facilitates the sedimentation of bacteria out of the flowing water column. The latter might explain the positive response of all α-diversity metrics and most of the abundant bacterial taxa to reduced current velocity in our experiment. The increased diversity at slower flow velocity, however, contrasts with those of previous related studies (Nuy et al., 2018; Salis et al., 2017).

3.5.2. Stressor interactions and changes in the functional profile

Each of our three stressors changed the functional profile of the microbial communities, supporting our third hypothesis (see further discussion below). Moreover, a range of complex stressor interactions were detected in the α-diversity metrics, the community relative abundances and the functional profile, as predicted in our fourth hypothesis. Both 2-way and 3-way interactions occurred frequently, confirming a high sensitivity of the benthic bacterial community to complex multiple-stressor effects compared to higher trophic levels such as stream invertebrates (Davis et al., 2018; Elbrecht et al., 2016; Salis et al., 2017). Bacteria have a much faster life cycle than invertebrates and can therefore respond quickly even to small changes in environmental conditions (Lau et al., 2015). Nutrient enrichment largely facilitated the microbial evolutionary response, i.e. more pronounced change in relative abundance, when added in combination with sediment or a reduction in flow velocity. On the other hand, sediment addition and flow-velocity reduction tend to hinder each other's, slowing down trajectories adopted when only one stressor was applied.

The stability of responses over time was evident from the unchanged direction across the two sampling dates (week 2 and week 3). Thus, benthic bacterial community responses were quite stable over time, adding more weight to the suggestion that they may be a useful tool for evaluating multiple-stressor effects on stream ecosystem processes (Nuy et al., 2018). Further, groups of bacterial taxa could be singled out in our SIMPER analysis because they explained the highest proportions of the variation from the control mesocosms. All these highly influential taxa were among the 13 most abundant taxa included in our MANOVAs, implying that focusing on these abundant taxa provided a realistic simplification of the complex bacterial assembly.

When combined, relative abundance changes in common bacterial taxa and predicted functions provide complementary information which can enhance our understanding of the general effects of stressors (Romero et al., 2019b) and enables us to postulate finer-scale hypotheses for more specific testing. For instance, the relative abundance of taxa known to degrade xylan and chitin increased when flow velocity reduction and sediment addition were combined. Both stressors facilitate deposition and retention of organic material on the benthic floor, which could indicate why functions translating into a higher capacity to process complex carbohydrates were enhanced. This interpretation is further supported by the increase in *Actinomycetales* and *Acidimicrobiales*, both members of the known organic matter decomposer

class Actinobacteria (Das et al., 2007), in the same treatments. The functional profile analysis also suggests changes in the metabolic pathways performed by the community. For example, in the sediment addition and flow-velocity reduction treatments, a reduction in chemolithoautotrophy can be anticipated due to an apparent decline in taxa capable of performing ammonia oxidation (METAcyc database). The growth of filamentous algae was greatly reduced in the unrestricted flow treatments (personal observation, N.P.D, Juvigny-Khenafou) reducing the availability of organic carbon sources, thereby likely favouring mineral pathways such as chemolithoautotrophy. We thus propose that the change in primary producer biomass limits the carbon supply to the detrital pool and the priming of the benthic bacterial communities (Halvorson et al., 2019). As a result, the bacterial community partially relies on different pathways to acquire their energy in those treatments; by contrast, when primary production, or other carbon supply, is favoured heterotrophic pathways are enhanced.

3.5.3. Non-random associations between the abundant taxa

Our network results indicate that non-random associations between dominant groups of bacteria exist validating our fifth hypothesis with phylogenetically related taxa such as Proteobacteria generally showing a higher number of potential biotic relationships. Members of microbial communities interact in a number of ways that affect their growth and metabolism, and these relationships can influence patterns of species abundance across space and time (Khan et al., 2019). Co-occurrence networks can illustrate how the growth of microbes correlate with both biotic and abiotic processes (Barberán et al., 2012). Despite their limitations (discussed in e.g. Carr et al., 2019; Freilich et al., 2018), co-occurrence networks have become widely used in microbial ecology to investigate potential patterns of competitive and cooperative evolutionary strategies, and to help develop subsequent targeted experiments. Phylogenetically related species tend to perform similar functions and respond to similar factors (Díaz et al., 2013). However, our results show that it is not unusual to find differences in the responses to multiple stressors between phylogenetically related and correlated taxa. Further, whilst some patterns may appear cooperative (positive correlations), others might suggest potential competition (negative correlations). The potential role played by biotic factors in multiple-stressor research has often been overlooked, even though such factors can help mediate community response to environmental factors (Steiner et al., 2005). For example, in our experiment highly connected taxa such as *Pseudomonadales* were also responsible for the highest proportions of the variability between stressor treatments (see SIMPER analysis).

Therefore, removal of these taxa from the assemblage could trigger unpredictable changes in abundance and functionality patterns, hampering the stability of the system (Tylianakis et al., 2010). However, the complexity of our benthic stream community did not allow us to determine if the relative abundance patterns observed for our highly connected bacterial taxa were the result of direct or indirect stressor actions. Consequently, our mesocosm-derived co-occurrence network should be complemented by subsequent laboratory assays in which the key groups of bacterial taxa identified in our experiment are studied in isolation (Carr et al., 2019). These laboratory experiments would increase our understanding of the relative importance of biotic and abiotic interactions in shaping the observed changes in bacterial community and functionality in the present mesocosm experiment.

3.5.4. Management implications and conclusion

Our mesocosm experiment has demonstrated that benthic stream bacterial communities are well-suited for detecting individual and interactive effects of multiple stressors within just 2-3 weeks. Further, in our study the complexity of the microbial community could be simplified and reduced to a small subset of highly indicative taxa. Given these encouraging findings, the rapid development of molecular techniques and the reduction of their cost, using benthic stream bacteria for biomonitoring purposes could provide a good alternative to macroinvertebrate-based river health indexes because the latter require more demanding sampling effort, longer laboratory processing time and expert taxonomic knowledge. However, the timescale of our experiment needs to be considered carefully when extrapolating our findings. Indeed, our experiment was performed over a short period of time with no spatial variation. Seasonality and geographical location are important determinants of microbial communities; therefore, repeated experiments across time and space are necessary (Lear et al., 2008; Taniwaki et al., 2019). A 1-year survey by Lear et al. (2009) of four streams impacted by varying degrees of human modification found that macroinvertebrate community structure changed clearly across the entire gradient of human impact while bacterial communities could only separate the most impacted site from the other three.

We propose that a combination of monitoring benthic microbial and macroinvertebrate communities could be used in the management of streams and their catchments, depending on the managerial objectives and more importantly the time of exposure to stressors. Whilst stronger long-lasting stressors may be identified consistently via microbial measurements,

weaker nuances may not, as a result of microbial resilience. Microbial biomonitoring thus may be more relevant in assessment of multiple stressors when the community is still in its acclimating to the new conditions. Finally, many benthic stream invertebrates rely on benthic microbial communities as food sources (Ayayee et al., 2018; Burgmer et al., 2010; Haglund and Hillebrand, 2005). Thus, changes in benthic bacterial communities in the early stages of stressor exposure could also be used to predict upcoming changes in invertebrate communities. In conclusion, combining the use of *in situ* manipulative experiments and molecular tools with laboratory targeted experiments may significantly improve understanding of how stream microbial communities respond to multiple stressors.

4. Chapter 4: Impacts of multiple anthropogenic stressors on stream macroinvertebrate community composition and functional diversity

4.1. Abstract

Managing ecosystems to ensure the provision of essential ecosystem services in systems affected by multiple stressors is a key challenge for theoretical and applied ecology. Trait-based approaches have increasingly been used in multiple-stressor research in freshwaters because they potentially provide a powerful means to explore the mechanisms underlying changes in populations and communities. Individual benthic macroinvertebrate traits associated with mobility, life history, morphology and feeding habits are often used to determine how environmental drivers structure stream communities. However, to date multiple-stressor research on stream invertebrates has focussed more on taxonomic than on functional metrics. We conducted a fully crossed, 4-factor experiment in 64 stream mesocosms fed by a pristine montane stream (21 days of colonisation, 21 days of manipulations) and investigated the effects of nutrient enrichment, flow velocity reduction and sedimentation on invertebrate community, taxon, functional diversity and trait variables after 2 and 3 weeks of stressor exposure. Deposited fine sediment and flow velocity reduction were the most pervasive stressors affecting invertebrate abundances and diversity, and their effects translated into a reduction of functional redundancy. Stressor effects often varied between sampling occasions, further complicating the prediction of multiple-stressor effects on communities. Overall, our study suggests that future research combining community, trait and functional diversity assessments can improve our understanding of stressor effects and their interactions in running waters.

4.2. Introduction

Freshwater ecosystems are experiencing extreme anthropogenic pressures. Almost all river catchments are influenced directly (e.g. via point-source pollution or physical changes) and/or indirectly (i.e. via global change) by human activities, reducing freshwater biodiversity and hampering effective ecosystem functioning (Allan, 2004; Davis et al., 2010; Heathwaite, 2010). The large number of simultaneously or sequentially operating stressors renders multiple-stressor studies a necessity in environmental research. However, such endeavours are not dominant in the scientific literature and a unified framework to mechanistically understand the effects of multiple stressors is yet to be proposed (Côté et al., 2016; Nõges et al., 2016; Schäfer and Piggott, 2018). Thus, lack of knowledge how stressors interact to shape ecological processes prevents stakeholders from making efficient short- and long-term managerial decisions for conservation, restoration or ecosystem services purposes (Lindenmayer et al., 2010).

Worldwide, stressors associated with land use changes such as urban development, water use, energy and food production have become particularly pervasive. They have accelerated biodiversity loss and ecosystem functioning declines via changes in the physicochemical parameters of streams, such as nutrient concentrations and ratios, levels of deposited and suspended fine sediment, flow velocity and water turbidity (Gordon et al., 2008; Horváth et al., 2019; Wu et al., 2019). Fine sediment reduces habitat heterogeneity by infilling the interstitial spaces in the stream bed, smothers the filtering and breathing apparatus of invertebrates and increases water turbidity (Piggott et al., 2015c). Nutrient enrichment, mainly N and P derived from fertilisers, often produces subsidy-stress response gradients in invertebrate communities (Wagenhoff et al., 2012; Woodward et al., 2012). Finally, water scarcity modifies the physical habitat and the diffusion of material and resources through flow velocity reduction (Calapez et al., 2018; Harvey et al., 2017a; Wu et al., 2019). One anticipated interaction between these stressors is that a reduction in flow velocity facilitates sediment deposition and, thereby, local accumulation of chemicals and nutrients whilst decreasing water re-oxygenation levels (Calapez et al., 2018).

To assess the effects of stressors on ecological stream health, benthic macroinvertebrate communities are often used (Bonada et al., 2006; Piggott et al., 2015c). Certain groups of invertebrates such as Ephemeroptera, Plecoptera and Trichoptera are highly sensitive to

changes in their physicochemical environment (Bonada et al., 2006). Invertebrates also connect streams across space and time, by dispersing over land and providing an important food source to higher trophic levels in both aquatic and adjacent riparian habitats (Sato et al., 2016).

One important concept in ecology is the 'environmental filtering' theory, which states that environmental conditions select for tolerant species and certain traits (Cadotte and Tucker, 2017; Poff, 1997). Whilst taxonomical assessments have often been used to assess the effects of multiple stressors on communities, they are bound to the regional species pool which reduces their potential for generalisation. To overcome this limitation, trait-based assessments, which rely on the compilation of community specific trait databases to characterise community niche breadth, have recently been getting more momentum (Ding et al., 2017). They highlight the functional significance of species, i.e. what they can do. They focus on the filtering role that environmental factors have in shaping community characterisations and provide mechanistic insights into community assembly and ecosystem processes (Poff, 1997; Statzner and Bêche, 2010; Wu et al., 2019). The results are not bound by the identity of species nor their regional pool but rather reflect phenotypic adaptations, thus facilitating the upscaling of local findings to larger geographical and longer temporal scales. In freshwater macroinvertebrate studies, traits associated with morphological characteristics, mobility, lifecycle, respiration strategy and feeding habits have been very informative (Dolédec et al., 2011; Poff et al., 2006). For instance, body shape and breathing apparatus are often associated with flow velocity and water oxygenation level (Calapez et al., 2018; Dolédec et al., 2011). The developmental pace of individuals mitigates their tolerance to stressors (Dolédec et al., 2011). Finally, feeding habits directly link to the metabolic and stoichiometric resources needed by the individuals (Cummins, 2016; Frainer et al., 2016).

Despite linking macroinvertebrate communities to wider ecosystem functions, trait-based assessments in multiple-stressor research tend to be restricted to observational rather than manipulative field experiments (Ding et al., 2017; Dolédec et al., 2011; Mor et al., 2019). Therefore, more data from multiple-stressor experiments conducted at the community and ecosystem levels in environmentally realistic scenarios are needed. To reduce this knowledge gap, we used field mesocosms to investigate the individual and combined effects of nutrient enrichment, flow velocity reduction and increased sedimentation on benthic macroinvertebrate communities and their associated functional traits. We aimed to determine if macroinvertebrate community stability is altered by stressor main effects and interactions through changes in

functional trait diversity. Based on the findings of previous related research, we tested three specific hypotheses:

(1) Sediment addition and flow velocity will have more pervasive stressor main effects than nutrient enrichment on community structure and trait composition because of their direct physical action on macroinvertebrates (Elbrecht et al., 2016);

(2) Nutrient enrichment will enhance the biomass accumulation potential, either via an increase in the mesocosms' carrying capacity or through a body-size shift towards larger organisms, due to increased resource availability (Cross et al., 2015; Frost and Elser, 2002; Ott et al., 2014);

(3) Interactions between flow velocity reduction and added sediment will be more common than interactions with nutrient enrichment (Elbrecht et al., 2016).

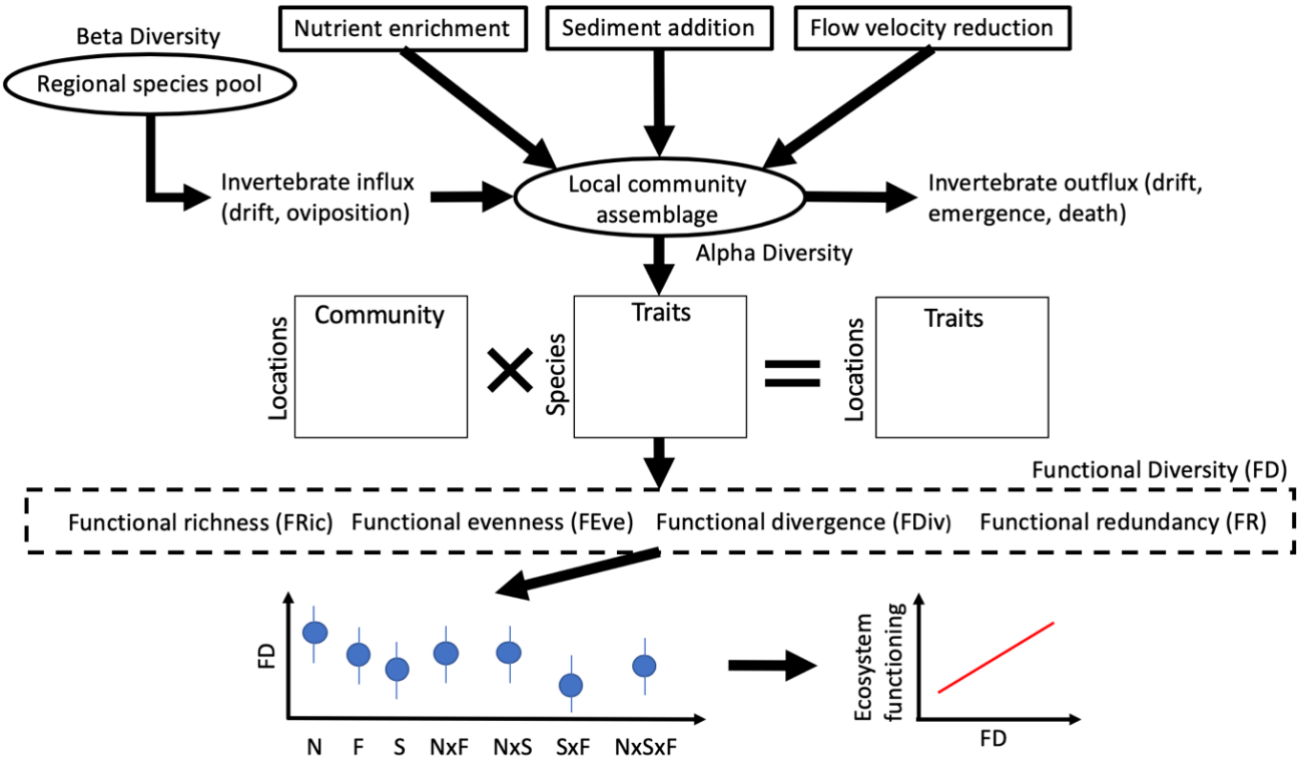


Figure 4.1: Conceptual model of the experiment. The benthic invertebrate community colonizing the stream mesocosms (regional community) is subject to different combinations of three stressors, nutrient enrichment (N), added fine sediment (S) and reduced flow velocity (F). The resulting, filtered communities (local community) possess different densities of traits, which then influence functional diversity of the ecosystem and its stability.

4.3. Materials and methods

4.3.1. Experimental mesocosm system

See Chapters 2.3.1, 2.3.2.

4.3.2. Experimental design

See Chapter 2.3.2 and 2.3.3.

4.3.3. Macroinvertebrate sampling

On each sampling occasion (Day 14 and Day 21), water flow was stopped in two header tanks and the whole substratum and the associated benthic invertebrates of the 32 mesocosms were sieved in the field using a 150-µm metal sieve and stored in 2-L PET containers. These were immediately filled to the top with 95% ethanol and later stored at -18°C in the laboratory until processing. After ~12 hours, one third of the ethanol was replaced with fresh ethanol to account for any dilution caused by the water remaining in the substratum. In the laboratory, the invertebrates were elutriated with a 450-µm sieve to remove the fine sediment and randomly divided into four equal subsamples. The specimen present in one subsample were counted, measured to the nearest 1 mm (body length excluding cerci and case, Piggott et al. (2015c) and identified to family using a stereomicroscope (Leica EZ4HD 8-35X, Leica microsystems GmbH, Wetzlar, Germany), except for Nematoda, Oligochaeta, and Acari (Brooks et al., 2011). When specimens could not be confidently identified to a family they were assigned to an order. We adopted this conservative approach across the whole dataset to reduce misassignments associated with the small size and general state of some specimens. Further, previous experiments suggest that family level of identification can be reliably used to examine community–environment and trait–environment relationships in aquatic habitats (Brooks et al., 2011; Tolonen et al., 2017). Adults in coleopteran families and dipteran pupae were kept as individual taxa as they present different biological characteristics from their larval counterparts. The remaining 3/4 of each sample was scanned for rare taxa, which were added to the total taxon count in each sample. We then extrapolated the total invertebrate abundance for all taxa in each mesocosm by multiplying the subsample counts by 4.

We measured 27 benthos-specific response variables: (i) total benthic invertebrate abundance, (ii) benthic taxon richness, (iii) Shannon's diversity index (H), (iv) Pielou's evenness index (J), (v) benthic EPT richness (number of taxa in the orders Ephemeroptera, Plecoptera and Trichoptera), (vi) benthic EPT abundance, (vii) three invertebrate size categories (following Piggott et al. 2015c), (viii) abundant taxa total community composition and (ix) abundances of the 17 most common benthic taxa, representing 95.1% of all individuals. We defined taxa as

being abundant if they represented 0.3% or more of all individuals and were present in at least 50% of all mesocosms (n = 32) (Beermann et al., 2018a; Elbrecht et al., 2016).

4.3.4. Species trait data

All invertebrates were assigned into five trait groups, which were subsequently divided into 22 trait categories, using a binary code (Li et al., 2019). Adult beetles (which were rare) and insect pupae were excluded from this classification. Selected traits featured lifecycle, habit, functional feeding groups, morphology, and respiration strategy (Table 4.1). Together these traits give an overall description of the ecological characteristics of the community and also represent aspects that are susceptible to having a close relationship with the manipulated stressors. Further, the traits provide information about the resilience and resistance of the community as well as more general biological characteristics (Ding et al., 2017; Dolédec et al., 2011; Li et al., 2019). Trait information was adapted from the literature (Ding et al., 2017; Merritt et al., 2008; Poff et al., 2006) and online databases (www.freshwaterecology.info). A summary of the different trait categories can be found in Table 4.1.

Table 4.1: Functional trait classification of the benthic macroinvertebrates in the mesocosms

Traits	Categories
Lifecycle	
Reproductive cycle	Semivoltine Univoltine Plurivoltine
Mobility	
Habit	Burrowers Sprawlers Clingers Swimmers
Morphology	
Body shape	Streamlined Not streamlined
Maximum adult size	Small (1 – 9 mm) Medium (9 – 16 mm) Large (> 16 mm)
Respiration	Brachial Intertegumentary
Foraging	
Functional feeding groups	Collector – gatherers Collector – filterers Scrapers Predators Shredders

We used the *dbFD* function in the FD R package (Laliberté and Legendre, 2010) to calculate the functional richness, functional evenness and functional divergence, as proposed by Villéger et al (2008). Together, these metrics can indicate whether species in an environment are performing similar (i.e. redundant) or different (i.e. complementary) roles for a given function or service (Wilkinson et al., 2018). Functional richness measures the amount of the functional trait space filled by a given macroinvertebrate assemblage (i.e. the set of species found in each mesocosm) irrespective of the species' abundances. Functional evenness quantifies the community's evenness in the functional trait space, whereas functional divergence measures the spread, i.e. divergence in distribution, of species relative to the centroid of the functional trait space (Chevalier et al., 2019). Finally, we also measured the functional redundancy as the difference between the Simpson's diversity (Pielou's) and Rao's quadratic entropy (Pillar et al., 2013).

We constructed a site × trait abundance matrix to represent community functional structure for each sampling unit. This matrix is obtained by multiplying a species × trait matrix by a site × species relative abundance matrix (Li et al., 2019). Only widespread trait categories occurring in at least 50% of all mesocosms were retained in this matrix to avoid introducing too many zero values. In total, we measured 24 trait-specific variables: (i) functional richness, (ii) functional evenness, (iii) functional divergence, (iv) functional redundancy, total trait composition in the community, and (v) 19 trait categories.

All data are available using the following link (to be replaced by the DOI after the viva date): https://figshare.com/s/0c49b2b9cce98d1b2138

4.3.5. Statistical analysis

All statistical analyses were performed using R (version 3.5.2, R Core Team). Where necessary, data were log-transformed to improve normality and heteroscedasticity after exploratory data analysis. We used a linear four-factor model with the same structure as Chapter 2.3.6 for all univariate analyses (all community-level response variables). The multivariate equivalent (MANOVA) of this model was used for the 17 common benthic taxa and the 19 widespread trait categories. The significance level and effect sizes were set as in Chapter 2.3.6.

4.4. Results

4.4.1. Community-level metrics

Total invertebrate abundance decreased with fine sediment addition. Abundance also decreased with flow velocity overall, but actually this effect occurred only after two weeks of stressor exposure (velocity × time interaction) (Table 4.2, Fig. S4.1). Total EPT abundance was also negatively affected by sediment addition and flow velocity reduction (Table 4.2). Total invertebrate taxon richness was unaffected by all treatments, whereas EPT taxon richness decreased when fine sediment was added. Lastly, Shannon's diversity decreased when sediment was added and Pielou's evenness showed a complex 3-way interaction (nutrients × sediment × flow velocity), with evenness being highest in nutrient-enriched mesocosms with reduced flow velocity but no added sediment (Table 4.2, Fig S4.2).

4.4.2. Body size metrics

Abundances of invertebrates in all three size categories decreased with sediment addition (Table 4.2). The effect of nutrient enrichment on small invertebrates (< 1 mm) changed from neutral after 2 weeks of stressor exposure to negative after 3 weeks (Fig. 4.2a). By contrast, nutrient enrichment increased abundance of large invertebrates (> 5 mm) on both sampling dates; moreover, fewer large individuals were found after 3 weeks than after 2 weeks (Fig 4.2b, Table 4.2). The effects of flow velocity reduction on small invertebrates was negative after 2 weeks but positive after 3 weeks (Fig. 4.2c). Finally, medium-sized invertebrates (1-5 mm) became rarer when flow velocity was reduced (Table 4.2).

Table 4.2: Summary (p-values and effect sizes) of linear model results of macroinvertebrate community-level response variables. For all manipulated factors, main effects are classified directionally as positive (+) or negative (–). P-values are in bold font where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.10$. Total invertebrate count = 37244.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Total invertebrate abundance	0.40	**<0.001** (0.40) –	**0.01** (0.12) –	0.29	0.83	0.07 (0.07)	0.92	0.43	0.12	**0.01** (0.13)	0.08 (0.06)	0.66	0.79	0.35	0.33
Total EPT abundance	0.64	**<0.001** (0.50) –	**<0.001** (0.27) –	0.25	0.56	0.31	0.055 (0.07)	0.52	0.37	0.22	0.31	0.26	0.77	0.69	0.27
Taxon richness	0.86	0.055 (0.07)	0.50	0.93	1.00	0.55	0.18	0.40	0.45	0.73	0.67	0.73	0.93	1.00	0.21
EPT richness	0.89	**0.007** (0.14) –	0.89	0.67	1.00	0.89	0.78	0.67	0.78	0.67	0.57	1.00	0.33	0.78	0.053 (0.07)
Shannon's Diversity Index	0.30	**0.02** (0.11) –	0.94	0.62	1.00	0.92	0.16	0.25	0.81	0.73	0.07 (0.07)	0.73	0.99	0.54	0.67
Pielou's Evenness Index	0.21	0.15	0.52	0.54	0.94	0.53	0.46	0.34	0.32	0.38	**0.01** (0.14)	0.88	1.00	0.50	0.56
Small (<1 mm)	0.104	**0.03** (0.10) –	0.13	**0.01** (0.12) +	0.49	0.32	0.56	**0.03** (0.09)	0.16	**0.008** (0.14)	0.18	0.11	0.68	0.84	0.30
Medium (1-5 mm)	0.32	**<0.001** (0.34) –	**0.02** (0.11) –	0.46	0.85	0.097 (0.06)	0.68	0.74	0.25	0.06 (0.07)	0.16	0.84	0.74	0.43	0.70
Large (>5 mm) (log10)	**0.005** (0.15) +	**<0.001** (0.30) –	0.87	**0.006** (0.14) –	0.44	0.46	0.37	**0.04** (0.08)	0.85	0.94	0.47	0.40	0.63	0.07 (0.07)	0.089 (0.06)

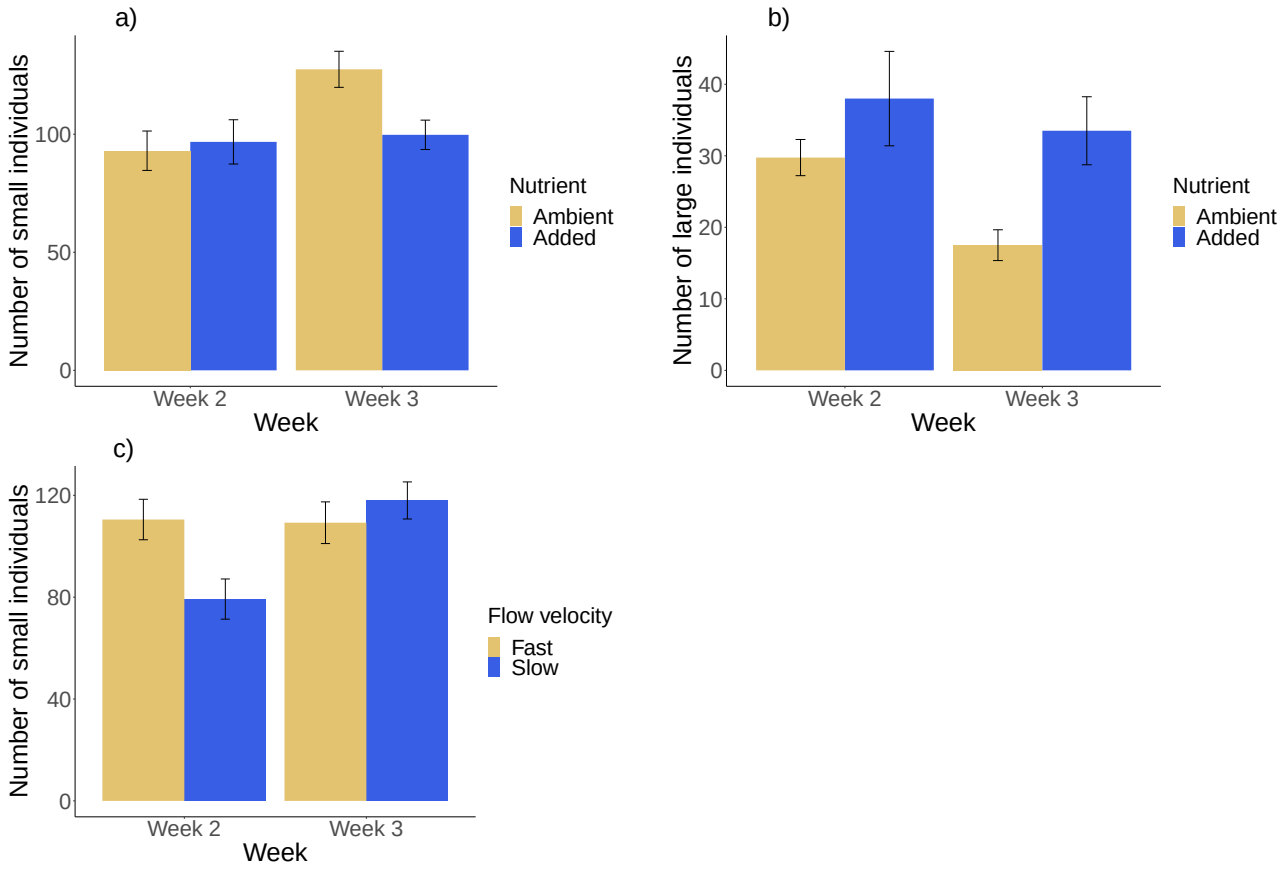


Figure 4.2: Average numbers per mesocosm of small or large benthic macroinvertebrates on the two sampling occasions showing the main effects of flow velocity reduction and nutrient enrichment (Error Bars = +/- SE, n = 32 per treatment).

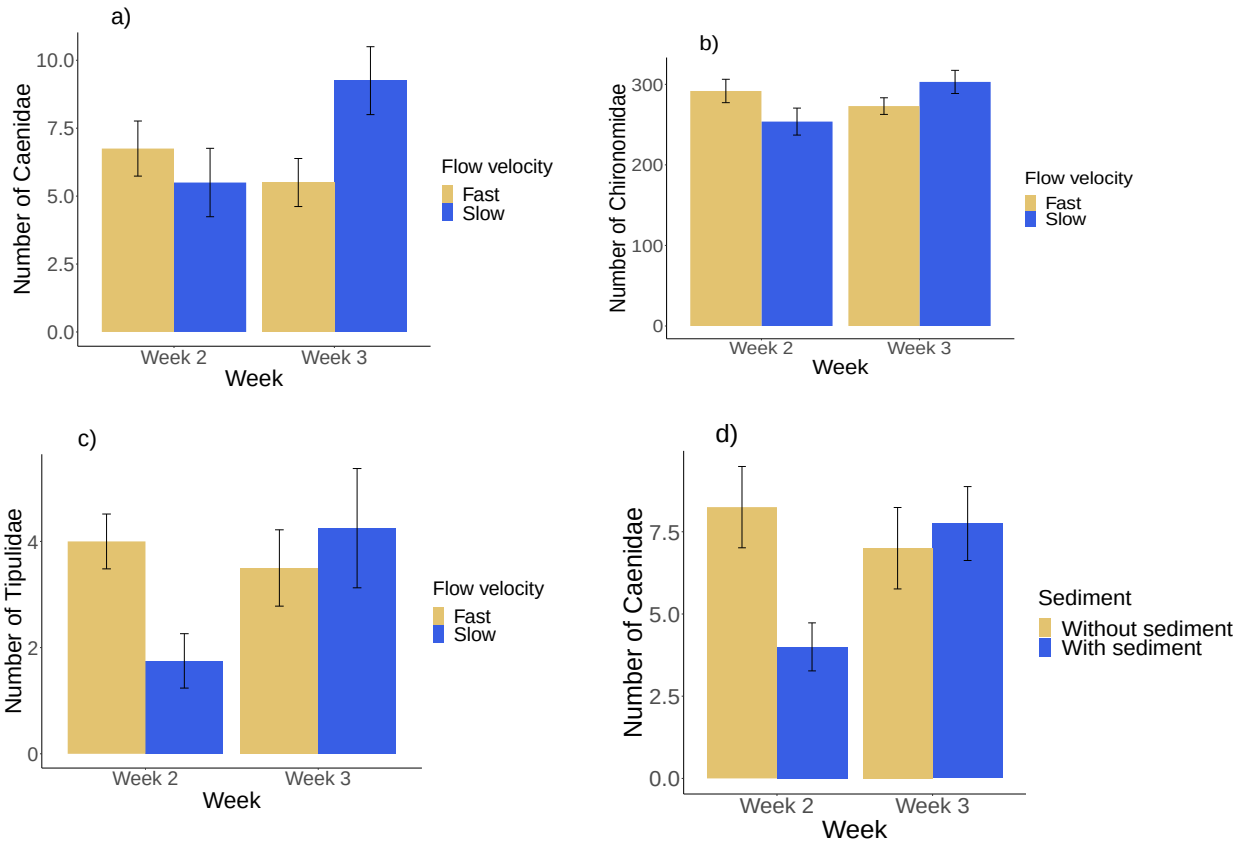
4.4.3. Common invertebrate taxa

The multivariate results of our analysis indicated that invertebrate community composition changed in response to added sediment and when the three stressors were manipulated together; community composition also changed from week 2 to week 3 (Table 4.3). Regarding taxon-specific responses, 41% of the abundant taxa (7 of 17) responded to at least one experimental factor as a stressor main effect. All seven taxa (the mayfly families Heptageniidae, Baetidae and Ephemerellidae, the dipterans Chironomidae and Tipulidae,

the caddis family Leptoceridae and the stonefly family Nemouridae) were affected by added sediment, followed by flow velocity reduction (Heptageniidae, Baetidae) and nutrient enrichment (Nemouridae). These ten stressor main effects were all negative (Table 4.3). Changes with time (independent of stressor effects) occurred for two taxa; Heptageniidae became generally more abundant after 3 weeks of stressor exposure whereas dipteran pupae became generally rarer.

Temporal changes in stressor main effects affected four taxa and occurred in six cases (3 for flow velocity, 2 for sediment and 1 for nutrients) (Fig 4.3.a-f). Caenidae, Chironomidae and Tipulidae all decreased in abundance after 2 weeks of exposure to flow velocity reduction (Fig 4.3.a, b & c). However, after 3 weeks their populations seemed to have adapted and increases in abundance were observed for all three taxa. For Caenidae, sediment decreased abundance 2 weeks after addition, but this negative effect had disappeared after 3 weeks (Fig 4.3.d, whereas the opposite temporal response pattern to sediment was observed for Tipulidae (Fig 4.3.e). Finally, Ephemerellidae increased in abundance when nutrients were added after 2 weeks, whereas after 3 weeks this effect had been reversed (Fig 4.3.f).

Interactions among stressors were almost as common as stressor main effects, affecting six common taxa (35%). Sediment × flow velocity interactions occurred for Baetidae, Nemouridae and dipteran pupae, nutrients × flow velocity interacted when affecting Ephemerellidae, and complex nutrients × sediment × flow velocity interaction occurred for Chironomidae and Gordiidae worms (Fig S4.7-8).



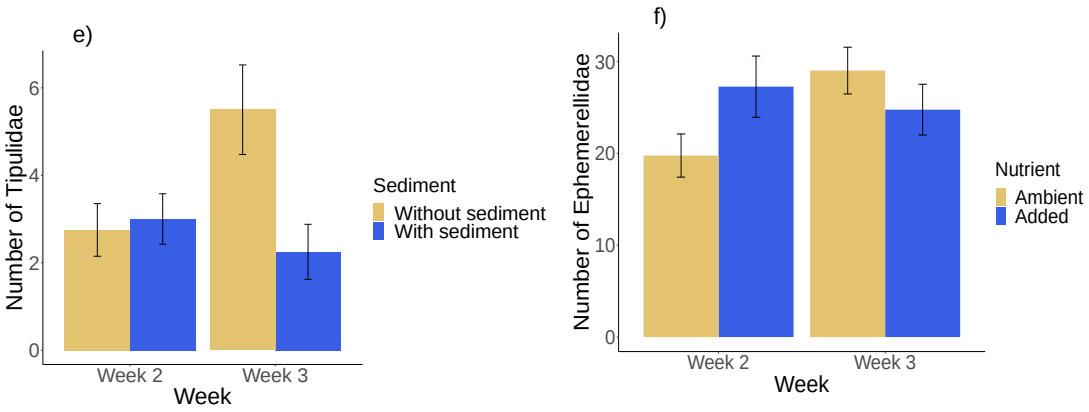


Figure 4.3: Average numbers of common invertebrate taxa affected by stressor main effects across both sampling occasions (Error Bars = +/- SE, n = 16).

Table 4.3: Summary (p-values and effect sizes) of multi- and univariate linear model results for the abundant taxa (with relative abundance). For all manipulated factors, significant main effects are classified directionally as positive (+) or negative (–). P-values are in bold font where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.1$. Total invertebrates count = 37244.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Community (95%)	0.45	**<0.001** (0.70)	0.06 (0.50)	**0.02** (0.55)	0.93	0.15	0.18	0.56	0.25	0.39	**0.02** (0.55)	0.13	0.44	0.98	0.97
Heptageniidae (6.9%)	0.17	**<0.001** (0.38) **–**	**0.02** (0.10) **–**	**0.03** (0.09) **+**	0.37	0.49	0.08 (0.06)	0.98	0.52	0.86	0.37	0.40	0.49	0.90	0.37
Baetidae (21.1%)	0.96	**<0.001** (0.36) **–**	**<0.001** (0.38) **–**	0.71	0.37	0.48	**0.046** (0.08)	0.60	0.08 (0.06)	0.17	0.37	0.21	0.60	0.56	0.35
Ephemerellidae (4.3%)	0.48	**<0.001** (0.32) **–**	0.96	0.15	0.22	**0.04** (0.08)	0.70	**0.01** (0.12)	0.12	0.09 (0.05)	0.96	0.09	0.12	0.87	0.96
Caenidae (1.2%)	1.00	0.10	0.24	0.24	0.48	0.81	0.64	0.24	**0.02** (0.10)	**0.02** (0.10)	0.64	0.16	0.06 (0.07)	0.81	0.10
Leptophlebiidae (0.6%)	0.24	0.15	0.38	0.15	0.56	0.25	0.38	0.56	0.77	0.77	0.56	0.25	0.56	0.77	1.00
Chironomidae (48.1%)	0.17	**0.01** (0.13) **–**	0.76	0.24	0.45	0.12	0.39	0.88	0.12	**0.01** (0.12)	**0.01** (0.11)	0.98	0.58	0.23	0.56
Empididae (0.4%)	0.71	1.00	0.46	0.46	1.00	0.46	0.27	0.46	0.27	0.27	0.27	0.07 (0.07)	0.71	1.00	1.00
Tipulidae	0.73	**0.04**	0.31	0.18	0.18	0.73	1.00	0.18	**0.02**	**0.04**	0.18	0.73	1.00	0.73	0.31

(0.6%)		(0.08) –							(0.10)	(0.08)					
Dipteran pupae (1.9%)	0.96	0.63	0.42	**0.01** (0.13) –	0.63	0.55	**0.03** (0.09)	0.26	0.12	0.55	0.55	0.63	0.55	0.42	0.42
Leptoceridae (1.7%)	0.40	**0.02** (0.10) –	0.61	0.50	0.18	0.13	0.74	0.18	0.87	0.18	0.50	0.07 (0.07)	0.50	0.24	0.87
Hydroptilidae (0.5%)	0.49	0.07 (0.06)	0.71	0.28	1.00	0.47	0.72	0.47	0.72	0.72	0.47	1.00	1.00	0.72	0.15
Elmidae (3.5%)	0.84	0.61	0.54	0.10	1.00	0.36	0.54	0.61	0.84	0.26	0.61	0.76	0.15	0.47	0.54
Perlidae (0.4%)	0.75	0.34	0.75	0.21	0.53	1.00	0.53	0.34	0.34	0.34	0.75	0.21	0.53	0.53	0.75
Nemouridae (0.6%)	**0.01** (0.12) –	**0.03** (0.09) –	0.39	0.60	0.60	0.23	**0.01** (0.12)	0.12	0.60	0.39	0.86	0.39	0.23	0.86	0.86
Acari (0.5%)	0.26	0.45	0.26	0.26	0.70	0.14	0.26	0.45	0.26	0.45	1.00	0.45	0.06 (0.07)	0.45	0.70
Gordiidae (0.5%)	0.29	0.14	0.06 (0.07)	0.83	0.29	0.83	0.06 (0.07)	0.53	0.53	0.83	**0.02** (0.10)	0.29	0.53	0.83	0.14
Nematoda (1.9%)	0.62	0.67	0.92	0.72	0.92	0.62	0.97	0.31	0.62	0.31	0.20	0.20	0.67	0.97	0.62

4.4.4. Functional diversity and traits

Two of the four functional diversity metrics were affected by sediment as a main effect, while none showed main effects for flow velocity reduction or nutrient enrichment. Functional evenness increased when sediment was added whereas functional redundancy declined (Table 4.4). Nutrients increased functional redundancy after two weeks of enrichment but not after three weeks (Fig. 4.4, Table 4.4), and redundancy also increased with time regardless of the stressor treatments. Further, functional redundancy showed a complex three-stressor interaction (Fig. 4.4). Nutrient addition increased functional redundancy at fast flow combined with added sediment but decreased redundancy at fast flow without sediment, whereas the opposite patterns occurred at slow flow.

The multivariate results of the trait analysis indicated that trait community composition (based on the 19 widespread trait categories) changed in response to sediment addition (Table 4.5); further, trait composition changed from week 2 to week 3 independently of the stressor treatments. Sediment (10 trait categories affected) and flow velocity (9) were the most pervasive stressors, followed by nutrients (2) (Table 4.5). Semivoltinism, plurivoltinism, burrowing and sprawling mobilities, predation, tegumentary respiration, non-streamlined morphology were favoured in the sediment treatments mesocosms whereas medium adult size, brachial respiration and streamlined bodies were hindered. Flow velocity reduction favoured univoltinism, borrowing and sprawling mobilities, predation, tegumentary respiration, and non-streamlined morphology. The opposite was found for swimmers, streamlined morphology and brachial respiration. Flow velocity deposition also triggered the only temporal response observed in the trait dataset with a positive influence on the abundance of shredders only observed after the first two weeks of exposure to the stressor (Fig S4.10). Finally, nutrient enrichment favoured the settlement of individuals with a large adult body size whilst reducing the smaller ones.

Interactions between stressors were not very frequent (16% less than for the common taxa) but all involved flow velocity reduction (Fig S4.11-13).

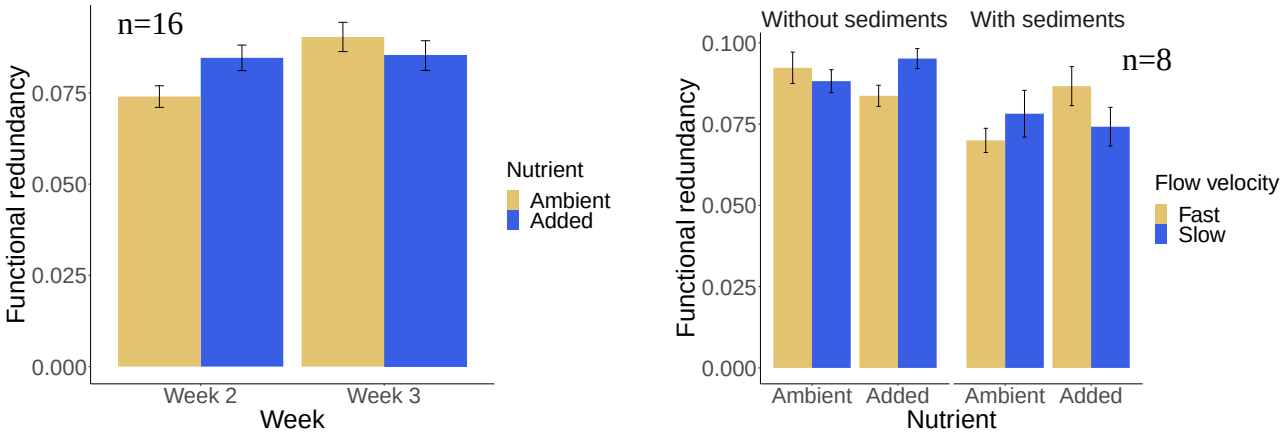


Figure 4.4: Nutrient main effects across sampling dates and three-stressor plots (averaged across both dates) for functional redundancy (Error Bars = +/- SE).

Table 4.4: Summary (p-values and effect sizes) of linear model results of the functional diversity measurements. FRic = Functional richness; FEve = Functional evenness; FDiv = Functional divergence; FR = Functional redundancy. For all manipulated factors, main effects are classified directionally as positive (+) or negative (–). P-values are bolded where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.1$.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
FRic (log10+1)	0.39	0.185	0.45	0.81	0.97	0.71	0.19	0.76	0.50	0.91	0.98	0.94	0.55	0.68	0.15
FEve	0.12	<**0.001** (0.24) +	0.33	0.42	0.99	0.29	0.17	0.62	0.62	0.1003	0.92	0.41	0.14	0.98	0.44
FDiv	0.82	0.09 (0.06)	0.37	0.41	0.86	0.88	0.73	0.54	0.73	0.68	0.13	0.65	0.17	0.47	0.68
FR	0.39	<**0.001** (0.25) –	0.81	**0.01** (0.13) +	0.26	0.69	0.36	**0.02** (0.11)	0.78	0.20	**0.001** (0.14)	0.65	0.91	0.11	0.39

Table 4.5: Summary (p-values and effect sizes) of multi- and univariate linear model results for the widespread trait categories (with percentage across all samples). For all manipulated factors, significant main effects are classified directionally as positive (+) or negative (–). P-values are in bold font where $p < 0.05$. Effect sizes (partial-η^2 values; range 0–1) are shown in parentheses for all cases where $p < 0.1$.

Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Sediment X Flow	Nutrient X Time	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Trait community (MANOVA)	0.053 (0.55)	**0.03** (0.58)	**0.17**	**<0.001** (0.74)	0.83	0.87	0.50	0.51	0.15	0.16	0.63	0.35	0.78	0.86	0.65
Semivoltine (4.4%)	0.46	**0.04** (0.09) **+**	0.51	**0.006** (0.15) **–**	0.80	0.72	0.86	0.90	0.14	0.59	0.65	0.54	0.23	1.00	0.48
Univoltine (6.8%)	0.41	0.27	**<0.001** (0.24) **+**	0.17	0.23	0.57	0.64	0.36	0.11	0.77	0.20	0.66	0.19	0.74	0.62
Plurivoltine (73.3%)	0.49	**0.01** (0.12) **+**	0.66	0.55	0.80	073	0.51	0.50	0.052 (0.08)	0.31	0.36	0.99	0.37	0.39	0.84
Burrower (50.4%)	0.71	**<0.001** (0.26) **+**	**0.003** (0.17) **+**	0.96	0.46	0.63	0.48	0.34	0.70	0.33	0.09 (0.05)	0.56	0.97	0.44	0.52
Sprawler (64.8%)	0.79	**<0.001** (0.21) **+**	**<0.001** (0.25) **+**	0.35	0.72	0.86	0.096 (0.06)	0.65	0.81	0.54	0.89	0.77	0.95	0.59	0.67
Clinger (89.4%)	0.43	0.51	0.08 (0.06)	0.20	0.53	0.63	0.07 (0.07)	0.25	0.59	0.57	0.15	0.84	0.95	0.79	0.94
Swimmer (25.8%)	0.80	0.051 (0.08)	**<0.001** (0.24) **–**	0.77	0.14	0.65	0.07 (0.06)	0.59	0.12	0.84	0.37	0.51	0.59	0.67	0.52

Streamlined (30.3%)	0.68	**<0.001** (0.22) **–**	**<0.001** (0.28) **–**	0.46	0.51	0.62	**0.008** (0.14)	0.72	0.69	0.35	0.84	0.76	0.77	0.76	0.47
Not streamlined (67%)	0.70	**<0.001** (0.22) **+**	**<0.001** (0.31) **+**	0.99	0.58	0.62	**0.04** (0.08)	0.93	0.99	0.34	0.81	0.68	0.68	0.83	0.41
Small (86.3%)	**0.03** (0.09) **–**	0.53	0.11	0.13	0.98	0.63	0.08 (0.06)	0.66	0.49	0.58	0.26	0.21	0.17	0.94	0.65
Medium (18.8%)	0.65	**0.001** (0.20) **–**	0.74	0.17	0.51	0.85	0.61	0.91	0.24	0.45	0.08 (0.06)	0.63	0.11	0.60	0.59
Large (1.7%)	**0.02** (0.10) **+**	0.63	0.22	0.48	0.98	0.33	0.92	0.89	0.41	0.73	0.91	0.83	0.85	0.66	0.11
Gatherer (81.9%)	0.78	0.106	0.77	0.55	0.53	0.91	0.42	0.78	0.36	0.17	0.59	0.19	0.78	0.51	0.68
Filterer (0.8%)	0.08 (0.06)	0.90	0.86	0.34	0.65	0.67	0.056 (0.07)	0.69	0.25	0.70	0.32	0.98	0.88	0.59	0.33
Scraper (15.8%)	0.51	0.08 (0.06)	0.91	0.75	0.68	0.78	0.52	0.49	0.35	0.94	0.43	0.27	0.97	0.36	0.59
Predator (53.6%)	0.73	**<0.001** (0.29) **+**	**<0.001** (0.22) **+**	0.68	0.57	0.54	0.38	0.58	0.78	0.50	0.21	0.17	0.98	0.43	0.65
Shredder (3.2%)	0.41	0.34	0.09 (0.06)	**0.004** (0.13) **–**	0.20	**0.02** (0.10)	0.34	0.72	0.35	**0.03** (0.08)	0.69	0.08 (0.06)	0.70	0.39	0.67
Brachial (44.1%)	0.80	**<0.001** (0.25) **–**	**0.01** (0.20) **–**	0.92	0.35	0.56	0.07 (0.07)	0.98	0.52	0.35	0.32	0.25	0.87	0.48	0.45
Tegumentary (53.9%)	1.00	**<0.001** (0.27) **+**	**<0.001** (0.26) **+**	0.79	0.38	0.48	0.24	0.75	0.86	0.44	0.29	0.18	0.78	0.59	0.39

4.5. Discussion

4.5.1. Stressor main effects on the invertebrate community

Our first hypothesis – sedimentation and flow velocity reduction have the most pervasive effects on macroinvertebrate community assemblage and trait composition – was supported. Moreover, all observed effects of both these stressors on invertebrate community-level metrics and abundances of common taxa were negative. This results differs from previous similar experiments which could be related to the fact that the source community for this experiment came from a truly "near-pristine" stream compared to agricultural stream elsewhere (Beermann et al., 2018a; Davis et al., 2018; Elbrecht et al., 2016; Piggott et al., 2015c). Fine sediment deposition caused a decrease in total invertebrate abundance irrespective of invertebrate size categories. We attribute this response to habitat homogenisation (Petsch et al., 2017), decrease in food availability (Matthaei et al., 2010) and physical damage to the breathing apparatus of gilled invertebrates (Piggott et al., 2015b; Wagenhoff et al., 2012; Wood and Armitage, 1997). The likely detrimental effect on brachial respiration is further supported by our univariate trait analysis, which displayed an increase in integumentary respiration concomitant to a decrease in brachial respiration when sediment was added. Further, abundance and richness of EPT taxa decreased with sediment addition overall, which was also reflected in the individual taxon responses. The lower abundances of these taxa in mesocosms with added fine sediment are likely due to increased emigration rates via drift and/or emergence (Beermann et al., 2018a; Piggott et al., 2015c).

Flow velocity reduction was the second-most pervasive stressor and displayed the largest number of changes with time in its effects on invertebrate community-level metrics and abundances of common taxa. Thus, the negative effect of flow velocity reduction after two weeks of stressor exposure on total invertebrate abundance and abundances of small individuals, Chironomidae, Tipulidae and Caenidae was no longer observed after three weeks. We suggest that interspecific microhabitat differences within these families led to an increased short-term drift response to reduced flow velocity, which was later masked by recolonisation of individuals belonging to different species within the same families that can tolerate or prefer slow flows (Harding et al., 2019; Zhang and Malmqvist, 1997). Previous studies have shown that flow velocity reduction often increases drift propensity, especially of swimming taxa, which is supported by our finding of fewer swimming taxa at reduced flow velocity (see

Functional diversity discussion below) (Beermann et al., 2018a; Piggott et al., 2015c). Further drift experiments should be done to confirm these patterns (Beermann et al., 2018a; Davis et al., 2018; Piggott et al., 2015c).

Our second hypothesis – enhancement of the biomass accumulation potential in response to nutrient enrichment either via increased carrying capacity or a shift towards larger-bodied organisms – was largely supported. Abundance of large-bodied individuals increased in nutrient-enriched mesocosms whereas small-sized organisms became rarer after 3 weeks of enrichment, despite total invertebrate abundance and community composition being similar (except for Nemouridae). Because immigration rates by drift into the mesocosms can be expected to be similar for all mesocosms (see Magbanua et al., 2013), this suggests invertebrate in nutrient-enriched mesocosms grew faster, with small individuals becoming medium-sized and medium-sized ones becoming large (Frost and Elser, 2002). Based on our findings, influx and outflux of medium-sized invertebrates more or less evened out, whereas outflux of large individuals exceeded the influx. Further, individual trait information indicates that nutrient-enriched mesocosms can harbour taxa with a larger maximum body size, supporting the idea that moderate nutrient enrichment act as a subsidy, favouring the growth of macroinvertebrates with higher metabolic requirements (Cross et al., 2003). Based on a related experiment by Wagenhoff et al (2012), our relatively low level of nutrient enrichment was probably already past the subsidy threshold for abundances of EPT and Chironomidae, two of the most abundant taxa in our experiment. Thus, we attribute the observed nutrient effects in our system to faster invertebrate growth rates combined with a capacity to support larger organisms rather than an increase in total carrying capacity.

4.5.2. Stressor main effects on functional diversity and trait categories

In agreement with previous studies, sedimentation and flow velocity reduction were key stressors driving functional diversity and trait category responses (Buendia et al., 2013; Calapez et al., 2018). However, because the colonising invertebrate species pool is the same for all experimental units and total taxon richness remained similar across all stressor treatments, it is not surprising that functional richness and dispersion were also unaffected by the treatments. Traits were neither ‘lost’ or ‘gained’, but rather relative abundances were shifted to reflect changes in the dominance patterns of the taxa best adapted to the new environmental conditions. Consequently, the reduction in functional redundancy associated

with sedimentation suggests that the community is more vulnerable to further 'functional loss' (Cummins, 2016; Pillar et al., 2013). Additionally, nutrient enrichment increased functional redundancy and seemed to stabilise it over time, probably by allowing species with similar resource needs to coexist via an increase in quantity and quality of resources (Piggott et al., 2015b; Sterner et al., 1993).

Shifts in feeding behaviours were also observed, with an increase in the relative abundances of predatory species when sediment was added or current velocity reduced. This result differs from Rabení et al. (2005) who reported a decline in total predator density when sediment cover increased, although these authors observed a broad predatory taxon-specific tolerance spectrum linked to their mobility. It is therefore possible that sediment deposition favoured individuals capable of burrowing or crawling without becoming prey themselves (Ding et al., 2017; Li et al., 2019; Rabení et al., 2005); our system lacked higher-order predators. Further, we speculate that reduced hiding space due to sediment deposition filling interstitial spaces in the mesocosm beds, combined with an increased mobility of sprawling predators, most likely facilitated their prey-catching success rate explaining their increased density under these conditions in our experiment. We also observed an increase in the relative abundance of shredders after two weeks of reduced flow velocity, which could be related to an increase in CPOM retention with velocity reduction (Death et al., 2009). In our experimental setup, CPOM variations are highly dependent on the source stream CPOM load fluctuations, which were not recorded but could help explain the temporal pattern observed.

Interestingly, trait responses to added fine sediment and reduced current velocity were often concomitant. For example, we observed a reduction in streamlined individuals, while the opposite response occurred for non-streamlined individuals associated with an increase in burrowing and sprawling individuals in mesocosms with both sediment addition and reduced flow velocity. Slower flow velocities facilitate sedimentation of fine particles but also make it easier for less aerodynamic organisms to move around. This suggests that, in our experiment, taxa adapted to reduced velocities also usually possessed features associated with increased sedimentation and *vice versa*.

4.5.3. Interactive effects on community and functionality

Interaction between two or all three manipulated stressors affected 6 of 17 common taxa and 3 of 19 widespread trait categories. Five of these interactions occurred between flow velocity and sediment, two between flow velocity and nutrients, and two were interactions between all three stressors, thus partially supporting our third hypothesis that interactions involving nutrient enrichment should be least common. Previous experiments have highlighted the importance of flow velocity and sediment deposition in shaping stream invertebrate community structure and functionality (Buendia et al., 2013; Dolédec et al., 2011; Elbrecht et al., 2016). Even though nutrient enrichment appeared to be relatively less important in shaping invertebrate responses in our experiment, past studies have shown that the effects of nutrient enrichment can differ strongly along an increasing gradient of concentration (Wagenhoff et al., 2012). Our enrichment treatment was fairly low compared to some previous similar experiments (Elbrecht et al., 2016); thus, it was perhaps not high enough (or long enough, at only 3 weeks of enrichment) to trigger strong responses of both community structure and trait composition. On the other hand, our enrichment might have already exceeded the subsidy threshold of our system, resulting in a decline in many response variables compared to their peak subsidy enrichment point (Wagenhoff et al., 2012). Distinguishing between the two outcomes would require further work involving a finer scale of nutrient (N + P) enrichment, to identify which side of the subsidy-stress gradient our results fall into.

In our datasets, interactive effects between stressors occurred most often in the abundance patterns of individual common taxa. Past experiments using the same stream mesocosm setup in Ireland and Germany also observed a similar trend (Davis et al., 2018; Elbrecht et al., 2016). In all three studies, moreover, EPT taxa were more sensitive than other taxa to two-way interactive effects between flow velocity reduction and either nutrient enrichment or sedimentation, as one might expect according to their high sensitivity to environmental changes (Bonada et al., 2006). The only taxon-specific three-way interaction observed in our experiment was a negative response of Chironomidae when exposed to all three stressors simultaneously. This result may seem surprising because this family is usually considered to be fairly tolerant to agricultural and urbanisation stressors (Li et al., 2019; Mor et al., 2019). However, we suspect this intricate three-way interaction to stem from the complexity of the Chironomidae family, which encompasses a diverse range of genera and species that vary widely in their microhabitat preferences and tolerance of various stressors. Thus, it is possible that whilst some midge species were more tolerant to one or two stressors, the overall family responded negatively to all three stressors combined. This results lends more weight to the

recommendation of Elbrecht et al. (2016) and Beermann et al. (2018a) that Chironomidae should be studied with a finer taxonomical resolution, for example by using DNA metabarcoding (Beermann et al., 2018b), to fully understand their response patterns to interacting anthropogenic stressors.

4.5.4. Trait and functional diversity vs. taxonomic and community-level metrics

Our experiment demonstrates the complexity of macroinvertebrate community dynamics and individual taxon responses to multiple agricultural stressors. Although traits and functional diversity showed a higher proportion of stressor main effects (74% of functional variables affected compared to 58% for community/taxon variables), invertebrate community and taxon responses were more sensitive to stressor interactions effects (31% vs 17%). Thus, taxonomical and trait approaches are highly complementary, even over short spatial and temporal scales (Cummings 2019). Whilst community abundance patterns can help us investigate macroinvertebrates dynamics, trait-based approaches give a mechanical indication of the reason why. Finally, functional diversity facilitates predictions about the stability of a given system to multiple stressors (Pillar et al., 2013). Further studies, ideally repeated over different seasons, spatial scales and incorporating ecosystem processes such as energy transfer between trophic levels, should be considered to improve our knowledge of macroinvertebrate community adaptation to multiple stressors (Kardol et al., 2018).

5. Chapter 5: Disconnected: How to integrate spatiotemporal complexity in riverine multiple stressor research

5.1. Abstract

Diverse anthropogenic stressors acting at the local, regional and global scale are reorganising species distributions, assemblages and biotic interactions over time and space. Although the importance of biotic interactions is acknowledged in shaping the species distribution and community structure, and tools to evaluate biotic interactions already exist, investigation approaches used in multiple stressor research have tended place more emphasis on abiotic drivers rather than biotic ones. Stressor research has thus implicitly focused on abiotic local selection processes filtering the density of species and their distribution. However, full abiotic selection (environmental filtering) is an extreme case along a continuum between abiotic and biotic drivers of biodiversity. The reality is more variable, and it has been argued that abiotic drivers alone cannot fully explain biodiversity patterns. For instance, well established biotic-abiotic feedbacks, such as how important species shape the physical environment then used by other organisms, also need to be incorporated into a more comprehensive modelling framework of multiple stressor effects. Exploiting the recent advances in inferring ecological networks, i.e. our ability to identify and quantify biotic interactions between species forming a community, we aim to demonstrate how incorporating biotic, spatial and temporal components into stressor research could increase our explanatory and predictive capacity and change how we implement conservation strategies. Network ecology, which is the structural description of the set of species interactions in an ecosystem, has already started to spread into the field of evolutionary biology and spatial dynamics. Because species linkages are important for ecosystem stability, and because the loss of these interactions could act as an early warning of instability, network theory could broaden our mechanistic understanding of multiple stressor interactions.

5.2. From a static to a dynamic multiple stressors research framework

The multidimensional response of communities to environmental changes and how it relates to ecosystem functioning are amongst the most central questions in ecology. Environmental stressors are the product of human activities and interact with each other, impacting communities and worsening the provision of ecosystem processes and services (Piggott et al., 2015b, 2015a). For instance, land use changes are often associated with new flow-velocity regimes, sediment loads or dissolved nutrient concentrations (Allan, 2004). Further, flow velocity reduction facilitates the local accumulation of fine sediment and chemicals whilst decreasing re-oxygenation levels (Calapez et al., 2018). Our current understanding of the effects of multiple stressors is largely focussed on the statistical properties of their interactions (synergistic, antagonistic or additive), and on their influence on aggregate community or ecosystem process measurements such as abundance, diversity or biomass accumulation (Bruder et al., 2019). In this context, the role of biotic interactions over time and space has been largely neglected despite feedbacks between community structure, stressors and biological parameters being well recognised (Bruder et al., 2017; Griffiths et al., 2018; Kraft et al., 2015). In parallel, the food web and network research community has recently been a very active think-tank in community ecology to understand species distribution, interactions and co-existence patterns (Barbier and Loreau, 2019; Barnes et al., 2018; Morales-Castilla et al., 2015). Simulation models of community assembly and several ways to incorporate biotic interactions have been proposed to investigate patterns of diversity, function and stability of ecosystems over spatial and temporal extents (Barbier et al., 2018; Kissling et al., 2012; Wisz et al., 2013). Further, in many cases a clear attribution of species distributions to environmental filtering (abiotic factors) is not possible (only in 15% of the cases) and it has been argued that biotic interactions play a stronger role in shaping the observed patterns (Kraft et al., 2015). However, biotic interactions have seldom been considered in multiple stressor studies. Effectively, there is a knowledge gap between the interactive effects of stressors and the underlying mechanisms across levels of biological organisation which calls for new modelling approaches or theoretical advances with a better integration of these so far distinct fields of ecology (Bracewell et al., 2019; Schäfer and Piggott, 2018; Thompson et al., 2018).

Community response to stress is implicitly linked to restructuring ecological networks stemming from changes in population densities, interactions and assembly processes. An ecological network approach to multiple stressors facilitates the identification of how

perturbations diffuse through the trophic assemblages and modify their functioning over space and time. It then informs on the direct and indirect pathways of multiple stressors effects (Calizza et al., 2019; O'Gorman et al., 2019, 2012). Thus far, multiple stressor research has disproportionally prioritised the investigation of change in density-weighted diversity indices in communities. However, feedbacks between biotic and abiotic drivers also affect population dynamics. For instance, the response of populations to stressors is modulated by biotic interactions. As an example, White et al. (2018) found that the identity of a predator mediated the response of community net productivity to both the single and combined effects of nutrient enrichment and warming. Lenihan et al. (2018) also found that predators modulated taxon-specific responses to stressor interactions. Equally, population densities modulate the abiotic parameters of an environment via ecosystem engineer species (Crain and Bertness, 2006; Jones et al., 1997; Wright and Gribben, 2017). These points acknowledge two things: that (1) not all trophic interactions are equal in holding communities together over time and space and consequently their response to multiple stressors (Tylianakis and Morris, 2017; Wootton and Stouffer, 2016), and (2) that the impact of stressors is likely to be disproportionate if affecting central or specialised nodes, triggering important trophic cascade reactions and rearranging the hierarchical organisation of communities (Griffith et al., 2018, 2017; O'Dowd et al., 2003). For instance, Ullah et al. (2018) showed in a marine system that a combination of warming and acidification changed the bottom trophic level towards cyanobacteria production which was then converted into detritus rather than biomass at upper trophic levels. By contrast, acidification alone enhanced the trophic flow between detritus and primary producer which translated into greater biomass accumulation in upper trophic levels (carnivores).

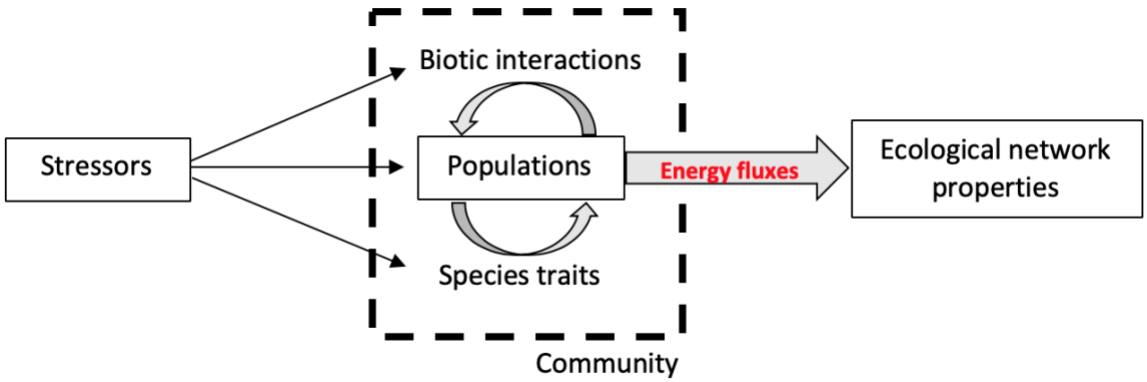


Figure 5.1: Diagram of stressor interactions in relation to community dynamics and organisational structure. The population density changes observed in response to stress are mediated by inter- and intraspecific biotic interactions which themselves depend on species functional traits. The trophic dynamics observed then influence how energy and resources are

made available to the different layers in the food-chain and thus regulate the emergent ecological properties of the system.

Further, biotic interactions are not fixed in space and time, neither are stressors and their levels. Rather, changes in biotic interactions underlie the adaptive capacity of populations to respond to multiple stressors. Whilst some species may have a broad tolerance to stressor gradients via behavioural, genetic, phenotypic or co-evolutionary responses, others more specialised may not (Gibert and Yeakel, 2019; Griffiths et al., 2018; Pauwels et al., 2010; Prunier et al., 2018). Altogether, empirical and theoretical progress in inferring biotic interactions, such as the use of eDNA, stable isotopes or machine learning and Bayesian frameworks, have rendered possible the construction of complex ecological networks over time and space, facilitating the upscaling of local responses (Godoy et al., 2018; Hutchinson et al., 2019; Morales-Castilla et al., 2015). They still remain to be largely validated empirically. Upscaling local responses is particularly important as diversity indices used in multiple stressor research do not upscale well over time and space. For instance, Chase et al. (2019) skilfully demonstrated how the magnitude of species richness change through time fluctuates across spatial scales. Further, community assembly patterns are modulated by the spatiotemporal scales used (Viana and Chase, 2019). Indeed, ecosystems are usually not isolated but rather connected through the exchange of resources and individuals in the landscape matrix over time (Gounand et al., 2018a). Therefore, indirect effects of stressors can diffuse over space and time; i.e what happens in one local patch is likely to influence the neighbouring ones by facilitating, or preventing, the movement of individuals (Tait and Larson, 2018). Equally, because of priority and legacy effects, observations made from one point in time to the next are likely to be linked (Baumgartner and Robinson, 2015; Busse et al., 2019). For instance, the presence of keystone species in an environment influences the spatiotemporal diversity of species. Booth et al (2019) recently reported that the diel movements of Sonora sucker fish (*Catostomus insignis*) are modulated by water turbidity fluctuations following discharge changes over time. The fish foraging behaviour then dictates the spatial arrangement of habitat heterogeneity directly impacting the invertebrate abundance and diversity over time. It becomes apparent that the spatiotemporal complexity needs to be embraced to reach the next level of understanding of the effects of stressors. Indeed community emergent properties (i.e the products of causal mechanisms, for instance species interactions, at lower levels of organization) such as network connectivity matter to emergent ecosystem properties such as net productivity (Newman et al., 2019; Tait and Larson, 2018).

In this chapter we use aquatic systems to outline how multiple stressor research can benefit from spatiotemporal considerations of biotic interactions to provide new insight into their effects. First, we assess how to give a temporal and spatial dimension to multiple stressor research using both spatial and trophic networks. Then, we explore how the spatiotemporal context can be used to develop new research questions and improve conservation incentives.

5.3. Spatiotemporal considerations in ecological networks under multiple stressors

Ecological networks are implicitly spatial, with higher trophic levels connecting lower trophic levels across space. Therefore, a consideration of spatial dynamics is essential in understanding how multiple stressors might affect the structure and functioning of ecological networks. Thus far for logistical reasons, most, if not all, multiple stressor manipulative studies have been done at a local scale (Mustonen et al., 2016; Piggott et al., 2015c). This focus on local scale conceptually narrows down the number of ways by which multiple stressors can influence biotic communities. For instance, most studies assume that all stressors occur at the same location and at the same time. Such assumptions greatly simplify the possible end point of the community structure that will either be an additive, multiplicative, dominant, neutral or reversal function of all the stressors considered. We argue that in many cases it is unlikely to happen this way in nature. Because most anthropogenic stressors are associated with land use change (IPBES, 2019), they are more likely to happen at different temporal scales and at landscape scale. For instance, a river flowing across a landscape would be influenced by different stressors along its length as a function of land-use change from upstream to downstream reaches. A local perspective in that case would suggest that one single stressor is affecting each portion of the river, but at the landscape scale, the structure of the riverine dendritic network and the species interacting and moving through that spatial network will influence how the effects of those stressors will unfold.

Stressors affecting the biotic community in the upstream reaches of a river network are likely to trigger spatial cascades by influencing biomass distribution in the local food web and thus ultimately the quantity and quality of the inorganic or organic material that will flow and subsidize downstream reaches (Gounand et al., 2017; Harvey et al., 2017a; Vannote et al., 1980). Those indirect spatial effects also include the passive or active movement of consumers

that could trigger important changes at the regional scale (Guzman et al., 2019; McCann et al., 2005; Pillai et al., 2011). For instance, Karatayev and Baskett (2020) showed that the arrival of external organisms in a system can modify the community trajectory following disturbance. Further, the introduction of exotic species can lead to change in the competitive patterns and trigger the collapse of whole trophic networks (Grosholz, 2005; O'Dowd et al., 2003). Ultimately the shape, particularly connectivity and modularity, of the spatial network will modulate how those spatial cascades play out and thus eventually drive the spread of the effect of those multiple stressors interacting across space (Gilarranz et al., 2017; Terui et al., 2018; Terui and Nishijima, 2019). Spatial networks are already known to constrain biodiversity patterns (Altermatt and Fronhofer, 2018; Carrara et al., 2012; Harvey et al., 2018), influence the spread of exotic invaders (Morel‐Journel et al., 2019), and affect ecosystem stability (Marleau et al., 2014). In a spatial multiple stressor context, unless one is dealing with a global stressor such as climate warming, there is a distinction to be made between the place where the stressor is introduced (point-source) and where its effects take place (stressor diffusion). We thus make a distinction between point source direct effects and diffuse indirect effects. Indirect effects require a vector to propagate, whether it is via the water flow or the movement of individuals (McClain et al., 2003; Schiesari et al., 2018). So far, for logistical reasons most of the multiple stressor research has focused on the direct effect of stressors (Juvigny-Khenafou et al., 2019; Manning et al., 2018; Mustonen et al., 2016). However, stressors are also likely to indirectly interact at central and well-connected nodes in the landscape away from their point-source (Figure 5.2; McClain et al., 2003). Those interactions would unfold mainly by the sum of the indirect effects with inorganic and organic suspended materials in the water columns varying in quantity and quality as well as species dispersing at varying density from connected patches under the influence of different stressors. The end result of those stressors interacting in space at those central nodes would be defined largely by species interactions with dispersing consumers influencing the strength of top-down trophic cascades and dissolved resources influencing the strength of bottom-up trophic cascades, with yet unknown effects on ecosystem functioning.

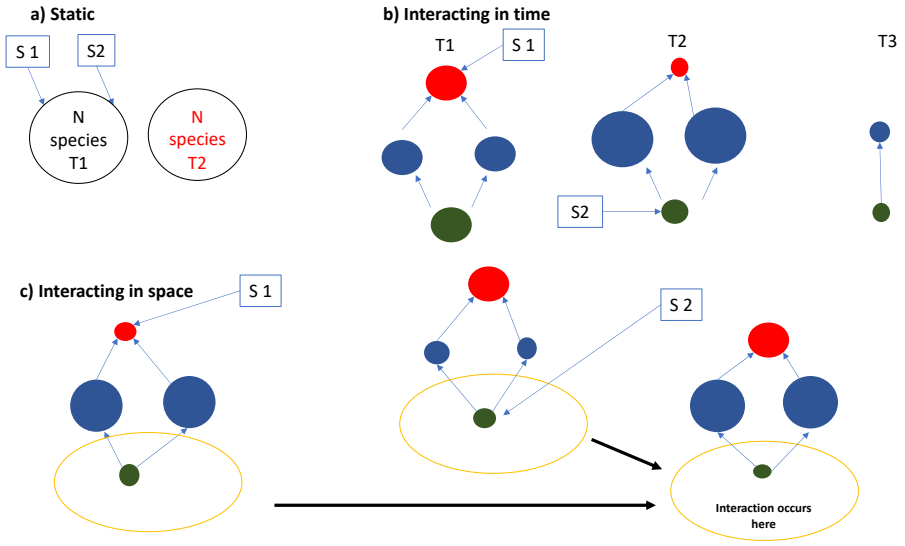


Figure 5.2: Stressor (S1 and S2) effects considering either (a) a static effect of stressors not influencing species richness over time (T1 -> T2), (b) temporally (T1 -> T2 -> T3) dynamic effect of stressors or (c) a spatially dynamic effect of stressors. In (b) the stressors are asynchronous and influence the density of distinct species in the trophic assemblage leading to a reduction in the food chain over time. In (c) stressors also influence the density of species but this time their actions occur in different patches resulting in a modification of species densities in downstream nodes.

In a spatial context, the same set of stressors could potentially lead to different endpoint communities and ecosystems. For instance, in a riverine dendritic network, the state of each downstream patch will depend on the nested accumulation of those multiple stressor interactions across the spatial network and will be defined by starting conditions, species interactions, abiotic interactions and the order at which the different stressors occur along the dendritic network. This means that the same stressors occurring in upstream reaches (e.g., wood harvesting, dam, agricultural pressure) could lead to a high degree of divergence in community structure and functioning among downstream patches. The fundamental difference with standard approaches to multiple stressors is that in a spatial network where each stressor affects single patches that are connected to more central patches, the interaction among the stressors will be mainly mediated by spatial dynamics of species, resources and other abiotic features and thus will be contingent on the nature of species interactions and energy pathways.

In a temporal context, the temporal distribution of stressor action, i.e. their frequency, order and magnitude, and the original community status matter throughout the river network by

creating varying levels of temporally structured heterogeneity (Baumgartner and Robinson, 2015; Mantyka-Pringle et al., 2019; Tucker and Fukami, 2014). How stressors influence the assembly processes depends on a threshold between niche (what species need), legacy (what the previous conditions were) and priority (the successional order of species arrival) effects as well as the degree of instability created by interacting stressors (Busse et al., 2019; Grainger et al., 2019). For river networks and biotic interactions this implies that the filtering effect stressors have on communities from one point in time to the next is modulated by the strength and the degree of asymmetry found in biotic interactions (Figure 5.3). Biotic interactions are not static, and organisms, most notably generalist organisms, can adapt to match the evolution of resources depending on their needs over time (Price et al., 2019). Species at well-connected and symmetrical nodes in a trophic network are more likely to be resistant to perturbations, irrespective of the order in which they occur, whereas overly dependent species are more prone to secondary extinctions (Pearse and Altermatt, 2013). In other words, species having a lot of trophic interactions can cope better with the loss of some of them. Similarly, in a two species interaction, a balanced symmetrical interaction, i.e. an interaction where species 1 depends and species 2 and species 2 depends on species 1, both with equal strength, implies that both parties are equally dependent on each other favoring stability. Further, the position of species in the trophic assemblage is likely to affect the resulting cascading effects from stressors temporal patterns with well-connected species more likely to spread the effect of stressors over time (Calizza et al., 2019). Whilst bottom-up and top-down population dynamic regulations are quite well understood for higher and lower trophic levels, central and connected species have the potential to generate both simultaneously. How the temporal diffusion of stressors unfolds depends on the strength of the synchronous relationships existing between dependent species as well as the magnitude of the impact. For instance, Sato et al. (2016) found that the effect of terrestrial invertebrate subsidies changed according to the subsidy timing in relation to the growing period of cutthroat trout (*Oncorhynchus clarki*). Further, long-lived organisms are more likely to persist over time in a stressed environment, if it is non-lethal, despite being functionally extinct, i.e. no longer reproducing (He et al., 2019). Additionally, individuals within species can have life-stage and life-history based sensitivity to stressors with younger individual often being more sensitive than older ones. Wagenhoff et al. (2011) found different stressors (sediment and nutrients) sensitivities between stream macroinvertebrates with different reproductive cycles (uni- vs multivoltines). Finally, the behavioral response of species to stressors may induce a temporal decoupling between the occurrence of stressors and the biological response. For example, many organisms can induce dormancy to 'weather the storm'

(Chesson, 1986). Once they resume their normal activity, they potentially can impact the other trophic levels e.g. increased grazing pressure or resource competition. Phenological changes in the development of stream organisms modifies, in theory, the degree of synchrony with their consumers (Sato et al., 2016).

High variability in space and time between localities can lead to divergent impacts of stressors. Thus, the way the stressors interact in the landscape and over time cannot only be understood from the usual statistical description of effects as being additive or interactive. Shifting the focus of multiple stressor studies from a time-fixed local perspective to a more continuous one such as discrete patches or reaches (Campbell Grant et al., 2007; Peterson et al., 2013), would help to bring the field into a more mechanistic framework that would make it easier to bridge with existing spatial and temporal ecological theory (meta-community and meta-ecosystem), to develop testable predictions. Much remains to be done but thinking about multiple stressors from a spatial and temporal perspective could lead to a re-definition of the way we generally think about stressor interactions and push the field toward a better understanding of anthropogenic influence on biodiversity and ecosystem functioning at landscape extent. In return, adopting an ecosystem approach to multiple stressors facilitates the development of transboundary managerial decisions which are currently limited.

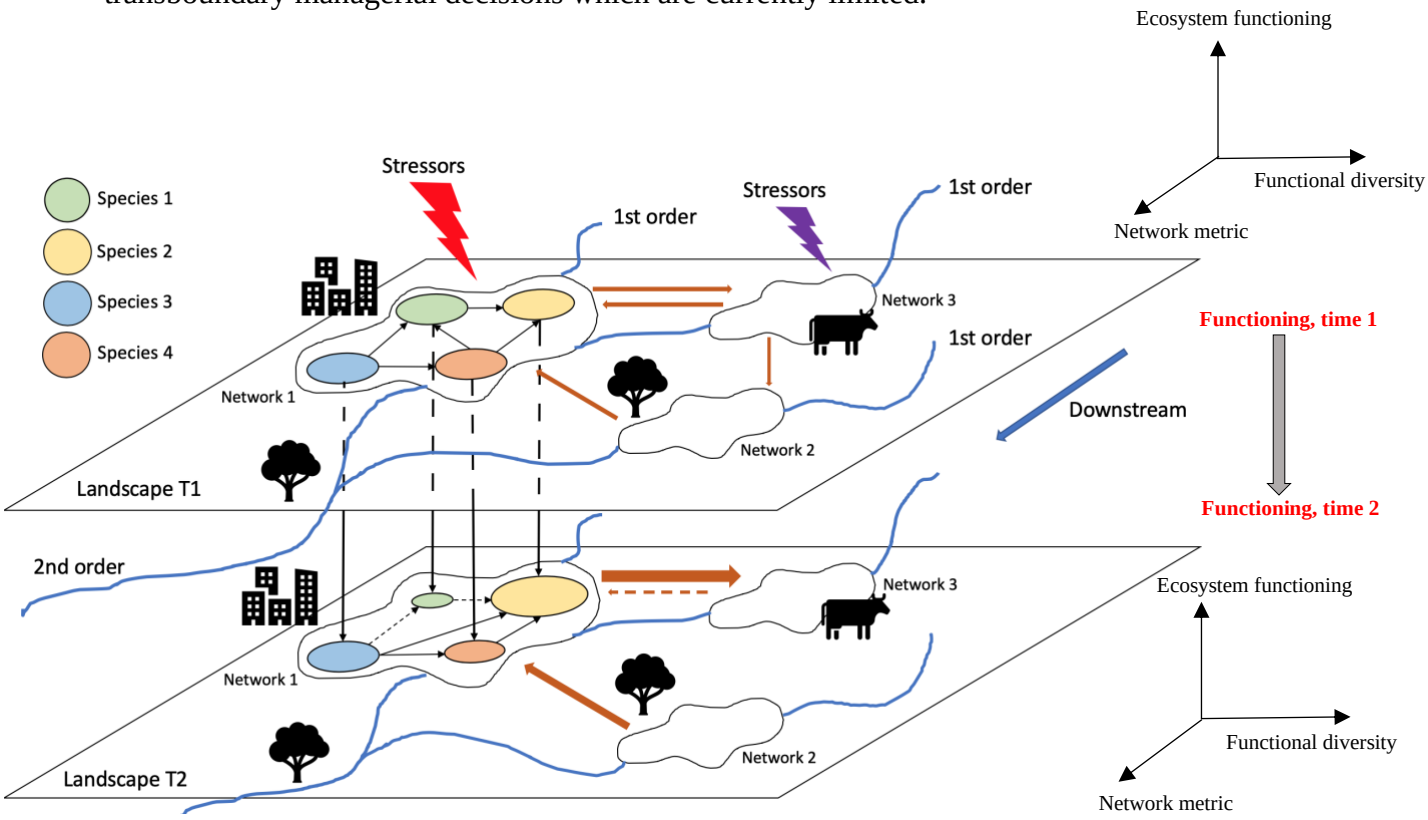


Figure 5.3: Landscape network representation of biotic connected in a river network. Interconnected biotic networks under stress undergo a rearrangement of their internal organisation as a local response to stress. This is usually translated in diversity changes and population density fluctuations. This reorganisation leads to a variation in inter-network exchanges reshaping the ecosystem functioning space through time; there are connections between patches either via the landscape matrix or the river network.

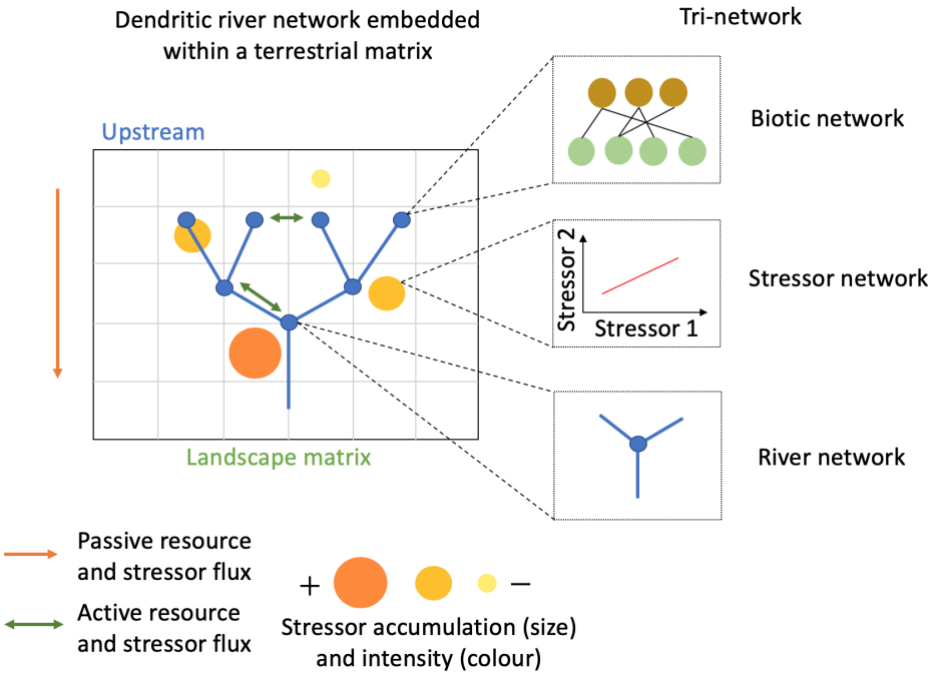
5.4. Adaptive bio-management of riverine systems

The high proportion of stressed versus pristine systems and the great diversity of stressors make the development of conservation strategies a priority (Craig et al., 2017; Harvey et al., 2017b; Mantyka-Pringle et al., 2016). Although we have accumulated a lot of specific knowledge on stressors and their impacts, we are not yet any closer to generating a global synthesis that is transferable into management actions. The high spatiotemporal heterogeneity in the communities, environment and stressor regimes complicate things greatly (Trøjelsgaard and Olesen, 2016). Equally, a case by case approach is neither viable nor cost efficient. Nevertheless, river and stream conservation incentives have never been more needed to protect freshwater biota and ecosystem services; the heterogeneity of stressors will continue to increase, and the transboundary nature of river and stream networks hinders the efficiency of local scale stressor management policies (Hering et al., 2015; Reid et al., 2019).

When managing environments, we aim to maintain or enhance their integrity by safeguarding their biological structure and functioning. Our approach to conservation usually assumes that once habitats are restored or protected, biodiversity will remain or come back. This is however very simplistic and there is compelling evidence showing that the evaluation of the success of conservation actions cannot be solely reduced to species or habitat inventories (Craven et al., 2018; Frainer et al., 2018; Mantyka-Pringle et al., 2016). Depending on conservation objectives the focus may change and a great deal of flexibility is required in conservation plans, with population densities connecting biotic networks through time and space (Hutchinson et al., 2019; Mantyka-Pringle et al., 2016; Perino et al., 2019; Raimundo et al., 2018; Ryo et al., 2019). We argue that by not placing enough emphasis on temporal, spatial and functional concepts, our conservation actions are limited and cannot accurately pinpoint where and when stability changes resulting from multiple stressors are in motion in the landscape matrix.

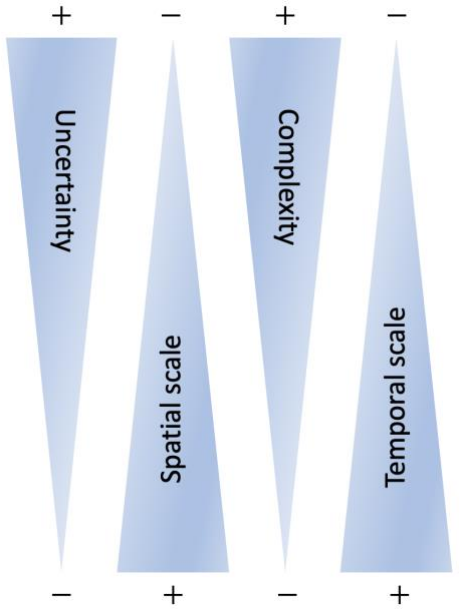
Bracewell et al. (2019) suggested to adopt a hypothesis driven network building approach, based on trophic linkage, to circumvent the aforementioned limitations and allow flexible conservation actions. Although it represents some improvements, it might rely too much on *a priori* knowledge. For instance, there are too many stochastic elements which cannot be accounted for when trophic interactions are pre-determined (Perino et al., 2019). Further, stressors also have their own regime and spatial distribution which influences the efficiency of conservation actions (Cardinale and Palmer, 2002; Davis et al., 2019; Perino et al., 2019). We suggest that the only way to unravel these changes, and to account for them in our modelling of stressors effects, is to track down the transfer of energy and material in the spatiotemporal heterogenous landscape matrix (Figure 5.4; Gounand et al., 2018b; Harvey et al., 2019; Kato et al., 2018). Indeed, empirical evidence shows that common 'ecological currency' pathways, such as O_2, C, nutrient budgets or stable isotopes, change under stress and underlie species traits and interactions (Kato et al., 2018; Price et al., 2019). Following this principle, ecological networks can be built and universally summarised into relevant metrics for conservation (Dormann et al., 2017; Hutchinson et al., 2019). By doing so, both the complexity of biodiversity assemblages and biotic interactions will be reduced and linked to ecosystem functioning in a framework applicable across systems in space and time, improving our understanding of multiple stressor effects.

1) Spatial relationships



2) Conservation applications

a- Trade-offs



b- Conservation action evaluation

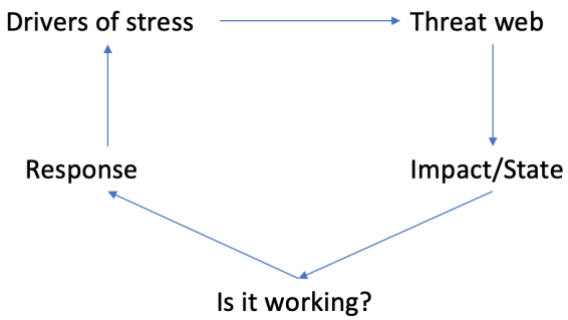


Figure 5.4: Tri-network approach and conservation trade-offs. The river environment is split in three layers with fluxes connecting different patches: the biotic component with the trophic networks, the anthropogenic component with the stressor correlation network and the abiotic component with the river network architecture. Conservation trade-offs can then weight in for each individual network layer (2a) to assess stressors effects at the different spatiotemporal scales and evaluate the efficiency of the different conservation actions (2b).

Additionally, management plans need to identify the most effective ways to alleviate the effect of multiple stressors especially when correlations exist. This point is often omitted from the current multiple stressor research directions (Mantyka-Pringle et al., 2016). For example, Geary et al. (2019) recently argued that to optimise conservation actions, understanding the relationships between co-occurring stressors through interaction 'threat webs' would help managers decide where to most efficiently apply their action. This gives an initial insight into how stressors are more likely to be present in the same landscape and how they might interact. Similarly to how changes in biotic interactions cascade through trophic assemblages, threat webs focus on correlations between co-occurring stressors to see how their regimes influence each other's across space and time benefiting the development of efficient mitigation strategies. Thus, we argue that in order to design effective conservation strategies, multiple stressor research needs to simultaneously have a dual approach: one focusing on the multiscale biotic interactions and ecosystem functioning and another one focusing conservation objectives and stressor mitigation strategies in order to derive maximum information to identify the mechanisms of species-environment (abiotic filter) and species-species (biotic filter) interactions in their natural dispersal/colonisation context (Figure 5.4).

There is a logistical trade-off between the degree of complexity that can be included in conservation schemes in a landscape context. Indeed, the difficulty of making sound assessments as well as interpreting results increases exponentially with the number of species and spatial scale. Recent spatial considerations advocate to include as many components of biodiversity as possible to understand spatial biodiversity patterns (Altermatt et al., 2020). Equally, our interpretation of stressor effects differs with spatial scale. What creates a strong instability at the local scale may be minimal in a regional meta-network with patch dynamics (Townsend, 1989; Weise et al., 2020). To synthesise the concepts explored in this paper, we propose a tri-network approach concentrating on (1) the relationships between network architecture changes and stressors accumulation, and (2) the conservation applications of network and meta-ecosystem concepts (Figure 5.4). The central aims are to: (i) identify the

relationships between stressors operating in the spatiotemporal boundaries and set targets; (ii) anticipate cost-effective strategies to remediate stressors to match the conservation targets; (iii) create trophic networks and identify the strength of interactions based on the observed flow of energy and matter; (iv) contextualise meta-networks in space and time to understand how communities and traits respond to the stressors; (v) evaluate the results of the implemented strategies and update overtime. We believe these different steps provide managers with the ability to modulate their actions over time and to implement pre-emptive actions as soon as changes are can be detected (Tylianakis et al., 2010).

5.5. Final remarks

The development of new analysis tools and theory is making network inference easier to us (Hudson et al., 2013; Poisot et al., 2016; Tikhonov et al., 2017). Combined with the parallel development of spatial analysis and monitoring techniques such as eDNA (Elbrecht and Steinke, 2019) the future of network theory integration into ecology and conservation studies is looking promising. After 20 years of field and laboratory exploration, we now have the technical tools, the empirical experience and the theoretical knowledge to push the boundaries of multiple stressor research. It's time to act on it.

6. Chapter 6: Synthesis and future work

6.1. Comparison among the three stressors

In this thesis the individual and combined effects of nutrient enrichment, fine sediment deposition and flow velocity reduction on pristine stream benthic communities were investigated. I conducted a single large-scale field mesocosm experiment and explored the responses of three key trophic levels supporting life in freshwater systems. I enriched nutrient concentrations to 2.19 ± 0.09 [SE] mg/L $N\text{-}NO_3^-$ and 0.12 ± 0.005 mg/L $P\text{-}PO_4^{+}$ compared to 0.57 ± 0.02 [SE] mg/L $N\text{-}NO_3^-$ and 0.01 ± 0.001 [SE] in the control units. I covered 100% of the benthic floor with 300mL of fine sediment and finally we set two flow velocities; fast (0.10 ± 0.008 [SE] $m.s^{-1}$) and low (below the detection limit of our instrument). All of the these treatments and levels are known to have an impact on benthic communities (Elbrecht et al., 2016; Salis et al., 2017). I also sampled at two different time scale; after two weeks of exposure to the stressors and after three weeks. In chapter 2, I focused on fungal and bacterial communities involved in the decomposition of leaf litter. This community was predominantly influenced by nutrient enrichment followed by flow velocity reduction; two factors known to modulate the activity of microbial decomposers (Table 6.1). This ranking of stressor impacts is in stark contrast with that from chapters 3 and 4, focussing on biofilm and macroinvertebrates respectively, which were most strongly influenced by fine sediment deposition followed by flow velocity reduction.

Table 6.1: Frequencies (in %) of significant responses of community structure and abundance of dominant taxa (with mean effect sizes; partial η^2 values, range 0-1; Garson, 2015) to the three stressors (nutrient enrichment, fine sediment deposition and flow velocity reduction) and time of exposure, as major effects and as interactions

	MICROBES (LEAF LITTER)	BACTERIA (BIOFILM)	INVERTEBRATES
NUTRIENT	21.2 (0.22)	17.4 (0.13)	8.2 (0.12)
SEDIMENT	0	87 (0.53)	53.1 (0.23)
FLOW	12.1 (0.15)	87 (0.46)	28.6 (0.23)
TIME	15.2 (0.13)	34.8 (0.18)	14.3 (0.13)
NUTRIENT X TIME	9.1 (0.09)	0	8.2 (0.1)
SEDIMENT X TIME	3 (0.08)	8.7 (0.09)	4.1 (0.1)

FLOW X TIME	3 (0.09)	13 (0.22)	12.2 (0.11)
NUTRIENT X SEDIMENT	6.1 (0.08)	56.5 (0.14)	0
NUTRIENT X FLOW	3 (0.08)	8.7 (0.10)	4.1 (0.09)
FLOW X SEDIMENT	0	87 (0.33)	10.2 (0.33)
NUTRIENT X SEDIMENT X TIME	15.2 (0.11)	0	0
NUTRIENT X FLOW X TIME	6.1 (0.10)	0	0
FLOW X SEDIMENT X TIME	0	0	0
NUTRIENT X SEDIMENT X FLOW	3 (0.09)	34.8 (0.10)	6.1 (0.12)
NUTRIENT X SEDIMENT X FLOW X TIME	9.1 (0.08)	13 (0.11)	0

This first cross-chapter comparison (Table 6.1) reveals that the target trophic level or ecosystem function used for the observation of stressor effects can influence how we interpret their importance. This shift in the importance of nutrient enrichment over the different chapters (leaf microbes > biofilm bacteria > invertebrates) reflects the direct and indirect effects that stressors have in a system (Halstead et al., 2014). For instance, dissolved nutrients are easily directly assimilated by microbes and algae (Battin et al., 2016; Danger et al., 2016). However, uptake of these nutrients by invertebrates is less direct, as the nutrients need to be fixed in tissues at lower trophic levels to then be assimilated via feeding (Frost and Elser, 2002).

Similarly, the importance of the sediment addition impact differed markedly among the three different ecosystem components (Table 6.1). Sediment is often considered as a master stressor negatively influencing all trophic levels (Blöcher et al., 2020; Lange et al., 2016; Mustonen et al., 2016; Wagenhoff et al., 2011). However, in the present experiment, only a thin layer of

sediment was deposited, and the alpha diversities and densities of abundant taxa across the different trophic levels were not as uniformly impacted (Table 6.1). Whereas invertebrates and biofilm largely followed the negative expected trends, the leaf decomposer microbes were unaffected by the sediment deposition (Table 6.1; Chapters 2,3,4). The direct/indirect mode of action is this time different to the response to nutrient enrichment observed in chapter 2. Sediment directly buried biofilm. But in contrast to the direct effect of nutrient enrichment on leaf litter decomposers, sediment had a less direct effect, as these microbes are largely found inside plant tissue, and are thus sheltered from the smothering and physical abrasion. The effect of sedimentation is expected to be indirect via an alteration of their resource exchange with the water column (Cornut et al., 2014). The lack of susceptibility to sedimentation by the decomposer microbes observed in our experiment indicates that unless the thickness of the benthic sediment layer is deep enough to create a clear physical barrier altering the diffusion of resources, leaf litter microbial decomposers remain largely unaffected by fine sediment deposition.

The biofilm communities (Chapter 3) were the most sensitive to any form of sediment deposition which most likely stems from their overall sessile nature and inability to remove the sediment cover smothering them. Numerous reviews have highlighted the importance of sedimentation in controlling biofilm communities by changing the physical property of the substrate (smaller surface area to attach) but also the resource exchanges (Battin et al., 2016; Besemer, 2015; Zeglin, 2015).

Invertebrates have been known to strongly react to sedimentation, selecting for species with traits capable of coping with this new environment, as observed in Chapter 4 (Dolédec et al., 2011; Wagenhoff et al., 2012). For instance, I observed an increase in tegumentary respiration in parallel with a decrease in brachial respiration in the treatments in which sediment was added. My results are therefore in accordance with observations in the field (Ding et al., 2017).

Flow velocity reduction has also been considered as a key stressor (Matthaei et al., 2010), but unlike the other two stressors its effects were observed on all three ecosystem components (Chapters 2-4). A reduction in flow velocity hindered the efficiency of microbial decomposition of leaf litter (Chapter 2), modified community structures and/or population abundances (Chapter 2-4), and also affected the relative distribution of specific traits in both microbes and macroinvertebrates (Chapter 3 & 4). These wide-ranging impacts, therefore, suggest that the hydrology of riverine networks may be a particularly important factor to be considered in multiple stressor studies (Widder et al., 2014).

Overall, sedimentation directly impacted two trophic levels (Chapter 3 and 4) and an average of 46.7% (0.25 effect size) of the community structure and abundance of dominant taxa across the three chapters. However, the effects of flow-velocity reduction were more widespread (Chapters 2 - 4) and slightly more pervasive although less biological variables responded on average (42.6%, 0.28). Sediment effects were more variable than the effects of flow velocity reduction. Indeed, across all of our treatments, higher and lower extreme values in the percentage of biological variables impacted as well as effect sizes were found in the sediment treatment (Table 6.1).

6.2. Stressor interactions

Stressor interactions were quite frequent across the different trophic levels, with the biofilm being the most susceptible to stressor diversity (Chapter 3). This susceptibility may be attributed to the mixture of trophic levels involved in biofilm development and consumption. Specifically, biofilms include a mix of autotrophic, heterotrophic and detrital food chains (Halvorson et al., 2019, 2017; Raghupathi et al., 2018). Biofilms fix resources from the water column and are highly sensitive to the abiotic parameters (Sabater et al., 2007). Autotrophic production is then a food source for the heterotrophic microbes and the whole biofilm material acts as a food source for the macroinvertebrates (grazers and gatherers). Later, dead biofilm and macroinvertebrate cells enter the detrital food chain releasing inorganic resources that can once again be assimilated by the autotrophic food chains and so on (Fig 6.1). All three stressors have the potential to affect any layer of this complex interactome. Sedimentation and flow-velocity reduction can modify microbial cell attachment to the substrate as well as resource exchanges linked with activity rates (Battin et al., 2016; Besemer et al., 2009, 2007). Nutrient enrichment mainly impact the activity rates of decomposition or primary production (Bruder et al., 2016b; Fernandes et al., 2014; Manning et al., 2018; Piggott et al., 2015b). Because of this high diversity of trophic interactions and the positive and negative feedbacks existing among the different components of the biofilm community, there are multiple scenarios which can generate an imbalance in the system. The imbalance in the flow of resources can then cascade up and down the trophic assemblage via multiple pathways to modify bacterial community structure.

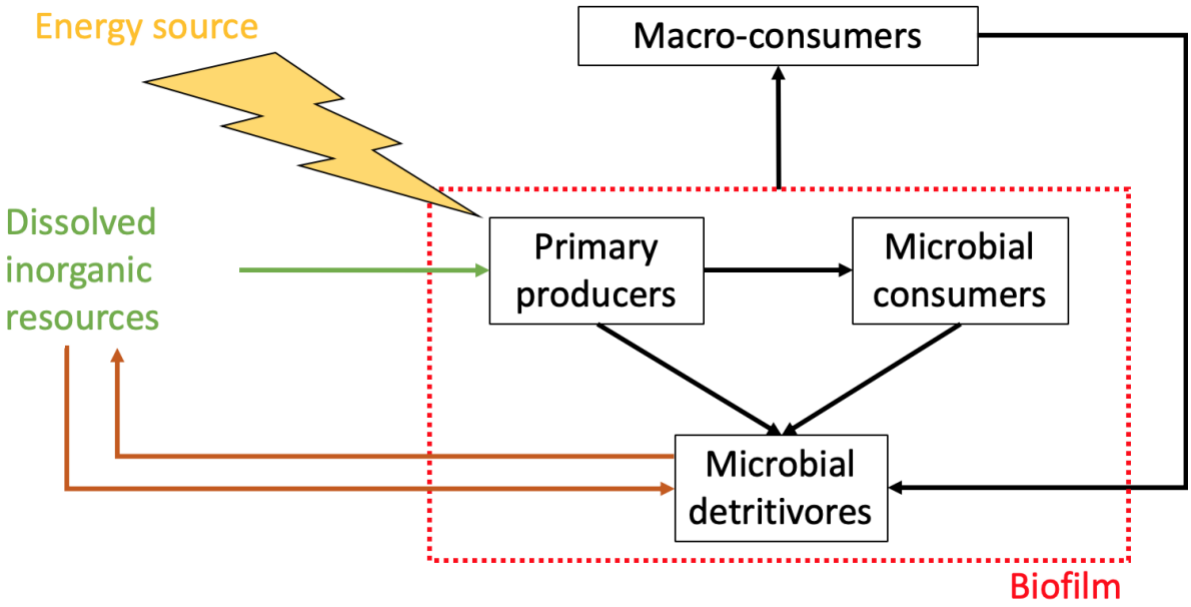


Figure 6.1: Trophic interactions among the different agents in biofilm community dynamics. The primary producers assimilate dissolved inorganic resources using an inorganic energy source before being fed on by the microbial consumers and the macro-consumers. Macro-consumers fed on the total biofilm material. Dead matter from both microbial and macro-consumers then enters the detrital pathway which releases dissolved resources.

On the other hand, the decomposer microbes (Chapter 2) largely feed on dead leaf litter (Gessner et al., 2010; Gessner and Chauvet, 1994; Kuehn, 2016). This resource is always present irrespective the stressor status. Organic leaf litter only fluctuates in abundance according to seasons (Gounand et al., 2018b) but in our experiment, I fixed this parameter with our leaf bags. Further, my experiment only occurred in one autumn when the riparian vegetation naturally shed its leaves and the microbial diversity in streams is expected to be at its highest. Additionally, similar to the main stressor effects, leaf litter microbial decomposers are sheltered from physical stressors: thus, I expected stressor interactions modifying the resource availability of microbial decomposers to be the most common interactions detected. This was confirmed in Chapter 2 with all 2- and 3-way stressor interactions involving nutrient enrichment (Table 6.1).

In our experiment, the susceptibility of macroinvertebrates to interacting stressors overall was qualitatively similar to that of the microbial decomposers (see interacting stressors in Table 6.1). However, flow-velocity reduction is the key parameter driving the susceptibility to stressor interactions rather than nutrient concentrations as in the microbial decomposers (Chapter 4; Table 6.1). This result indicates that a small difference between faster and reduced

flow velocity can have large impacts, highlighting the importance of flow regimes in modulating invertebrate communities. Placed in a global change context this importance is particularly relevant, as managerial measures altering flow regimes are expected to increase, but also climate variations in temperature and precipitation patterns are likely to severely change river flow patterns (Gordon et al., 2008; Lange et al., 2018; Suen, 2010). Furthermore, from our three experimental data chapters, I deduce that community structure of biofilm is more prone to changes under stressor accumulation compared to that of microbial leaf decomposers and macroinvertebrates. Biofilms are often considered as the 'skin of rivers' (Battin et al., 2016). Thus, following this logic important changes in composition and structure could have significant consequences for the entire lotic ecosystem. The position that biofilms occupy in stream trophic network assemblages makes them particularly important, as a change in their activity could rapidly trigger bottom-up and top-down trophic cascades via changes in primary production (energy source for both higher trophic levels but also decomposers), whereas in our experimental system I anticipate macroinvertebrates to generate top-down cascading trophic reactions and leaf litter decomposition producing bottom-up effects (Ullah et al., 2018).

6.3. Temporal patterns

The main effects of the stressors often changed over time and sometimes so did multiple stressor interaction effects. Specifically, the main stressors effects changed over time in at least one response variable except the nutrients on biofilm (Chapter 3, Table 6.1). Several mechanisms could explain these changes over time depending on which trophic level is considered. For the microbial communities (Chapters 2 & 3) this effect may be the result of successional changes occurring over short temporal scales (Duarte et al., 2010; Wey et al., 2012). However, for the macroinvertebrate community (Chapter 4) the change over time is likely to be due to intraspecific immediate response followed by phenotypic adaptive plasticity (De Laender et al., 2014; Musseau et al., 2019). In our experiment, adaptive responses between week 2 and week 3 that influence community dynamics likely include behavioural adaptations (induced drift) (Elbrecht et al., 2016) and phenotypic variability favouring individuals within species with certain traits (Zhang and Malmqvist, 1997).

Furthermore, my thesis emphasises that to understand the effect of multiple stressors on biodiversity we cannot restrict our assessment to single time points and finer successive

temporal measurements should be conducted (Chapters 2-5). Indeed, as mentioned in section 6.2, stressors have direct and indirect effects which operate over different time scales. Whilst direct effects occur immediately, indirect effects may need a longer time period to be recorded. Placed in an ecosystem context, indirect effects may upset the synchrony in population dynamics underlying ecological processes (Townsend, 1989). In return, upsetting population dynamics could jeopardise the long-term stability of the system and increase its susceptibility to further stressor events or taking it into a new intermediate stable state (Gordon et al., 2008; Karatayev and Baskett, 2020). Temporal questions in stressor regimes also raise the question of chronic vs acute stress events as well as their variance (Davis et al., 2019). Variance and frequency have less often been incorporated in stressor research and deserve more consideration (Benedetti-Cecchi, 2003; McCabe and Gotelli, 2000). Furthermore, as indicated in Chapter 5, spatial and temporal mismatches between stressors need to be investigated further. Indeed, in a natural context many stressors are likely to be temporally and spatially disconnected, however they can still be interacting in downstream river segments (Fagan, 2002; McClain et al., 2003). For instance, chemical stressors get carried along the river network via the river flow and interact with other stressors at connection nodes between distinct river branches (McClain et al., 2003; Schiesari et al., 2018). As a result, the spatial pattern of disturbances is likely to have a strong effect on population connectivity in river network (Campbell Grant et al., 2007).

6.4. Merits of taxonomic and functional diversity assessments over time

As observed in Chapters 2, 3 & 4, the high variability in the responses to stressors, both singly and in combination, makes it difficult to generate a general model of stressor effects, especially as the trophic complexity increases. However, by combining functional and taxonomic assessments at multiple trophic levels we are able to better reveal the mechanisms of stressor responses at the population level and extrapolate to ecosystem functions (Chapters 3 & 4). Indeed, understanding the effects of trait variability on ecological mechanisms is a fundamental goal of functional ecology (Musseau et al., 2019). Equally, understanding population dynamics in response to environmental parameters is a tenet of population ecology (De Laender, 2018). Combining functional and community ecology in stressor research may allow us to make more accurate interpretations of species coexistence and changes in activities leading to ecosystem processes (Cummins, 2016).

Biodiversity assessments have become a global political priority (IPBES, 2019) and are also closely linked to ecosystem functioning measurements in ecological research (Biodiversity – Ecosystem Functioning theory, B-EF; Naeem et al., 1994; Tilman and Downing, 1994). However, biodiversity assessments tend to rely a lot on aggregate metrics such as alpha, beta and gamma diversities. To encompass the complexity of stressor interactions, aggregate metrics appear to be not fully appropriate (Chapters 2 – 5). Indeed, whilst alpha diversity overall displayed fewer significant effects, the work of this thesis indicates complex responses in the individual abundant taxa with temporal variability. Further, stressor studies can be highly context dependent depending on which taxonomic group is investigated as shown in this thesis. Additionally, different regions have different species, different abiotic parameters and different stressors and stressor levels, which can result in further variability of outcomes in multiple stressor research. The underlying concept of the B-EF is that an increase in taxonomical diversity increases functionality (Soliveres et al., 2016). However, as seen in this thesis, significant changes in function or functional traits can be observed in the absence of taxonomical diversity changes (Chapters 2 & 4). Thus, I argue that the strength of biodiversity assessments is only proportional to the extent to which functional diversity is also considered (Fonseca and Ganade, 2001).

6.5. Management implications and future research orientations

The amount of known and unknown stressors influencing riverine network communities is too high to make it logistically feasible to evaluate the responses of aquatic communities to each individual stressor and their combinations (Dudgeon et al., 2006; Halstead et al., 2014; Reid et al., 2019). Despite this need, research has largely described the emerging properties rather than been mechanistic (Halstead et al., 2014). In this thesis I aimed to combine current trending quantitative approaches to investigate the biodiversity response to interacting stressors with field manipulative experimentation. By combining molecular with taxonomic and functional approaches it may be possible to develop a more integrative platform to provide river health assessments (Altermatt et al., 2020). In particular, molecular approaches can be used to sample all trophic levels simultaneously. Whilst there are still technical pitfalls that need to be addressed, I suggest that the scientific community could also focus on how molecular techniques currently complement existing biomonitoring practices. One obvious example is that for many taxa, such as Diptera, taxonomic assessment can be difficult and prone to errors (Beermann et al., 2018b). Further, molecular techniques also enable the assessment of

functional genes and their expression (Vacher et al., 2016). Therefore, where multiple laboratory assays are needed, such as the enzymatic assays of microbial activity (Sinsabaugh and Findlay, 1995), molecular techniques allow the simultaneous mapping of taxonomic and functional diversity found in stream communities. As their cost continues to reduce, molecular techniques represent promising tools that enable fast and extensive sampling schemes with fewer specialist staff.

One key message of this thesis is that trophic levels display a high variability in how they react to stressors, both individually and combined. Another message is that temporal scale is an important factor in determining how stressor effects are perceived. Finally, there is a need to combine biodiversity measurement with functionality measures of communities. Therefore, I advocate that decision-makers adopt an ecosystem approach to effects of multiple stressors on river and stream biodiversity and health, sampling all trophic levels (or at least as many as possible) (Altermatt et al., 2020; Barnes et al., 2018; Gounand et al., 2018a). Indeed, most monitoring schemes focus more specifically on target groups such as Ephemeroptera, Plecoptera and Trichopetra (EPT) (Bonada et al., 2006). However, the extrapolation of the responses seen in these orders is far from being suitable to other taxonomic units, and within-order variations also exist (Altermatt et al., 2020). Further, sampling different taxonomic units, such as invertebrate, fish or microbes, requires different techniques, spatial scales and sampling effort, which can introduce multiple biases in multi-trophic assessment. There is often a mismatch between the life-cycle of populations and the adopted spatiotemporal sampling strategy adopted in stressor research where sampling is often conducted over short period of time and spatial extent (Altermatt et al., 2020; He et al., 2019). Whereas microbial communities exhibit changes over hours and days with hundreds of generations at the local scale, it can be a much slower process for invertebrates and vertebrates, across a wider spatial scale; up to several years with much fewer generations over several kilometres (He et al., 2019). However, monitoring schemes often tend to be done over timeframe ranging from months to years and most multiple stressor studies only consider a single time point. Maybe a nested temporal approach where multiple successive fine spatiotemporal scale recording repeated at intermediate frequencies over a long period of time would help fill that gap. Further, because of the directed connectivity existing in riverine networks there is a spatial autocorrelation element to be considered (Fagan, 2002).

Altogether these current methodological problems make it difficult to extrapolate of low- or inadequate resolution effects on biodiversity to whole ecosystems and to larger spatiotemporal scales difficult: one ends up comparing ‘oranges to apples’, making it hard to understand biodiversity patterns in changing environments (Chase et al., 2019; Viana and Chase, 2019). Therefore, in order to drive multiple stressor research forward, spatial, temporal and manipulative studies should become more dominant in the research agenda to untangle multiple stressor effects originating at different times and places, but still have important combined effects such as heavy metals accumulating in biomass and being transported to be released in different places or at different times via a vector (McClain et al., 2003; Schiesari et al., 2018).

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Appendices

Chapter 2

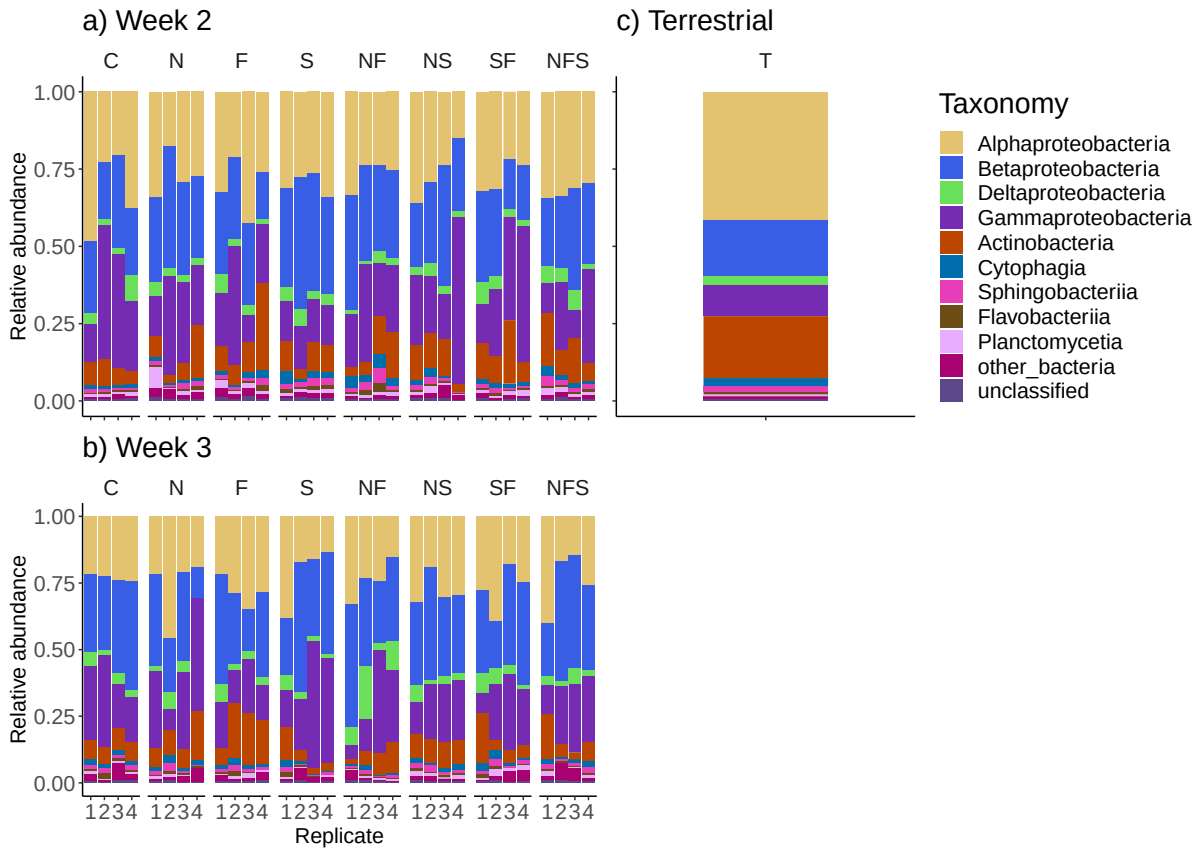


Figure S2.1: Relative abundances of the bacterial assemblages in decomposing leaf material at the class level after senescence, 2 weeks and 3 weeks of stressor exposure. C: Control; N: Nutrient enrichment; F: Flow velocity reduction; S: Sediment addition; NF: Nutrient + Flow; NS: Nutrient + Sediment; SF: Sediment + Flow; NFS: All three stressors; T: Terrestrial litter

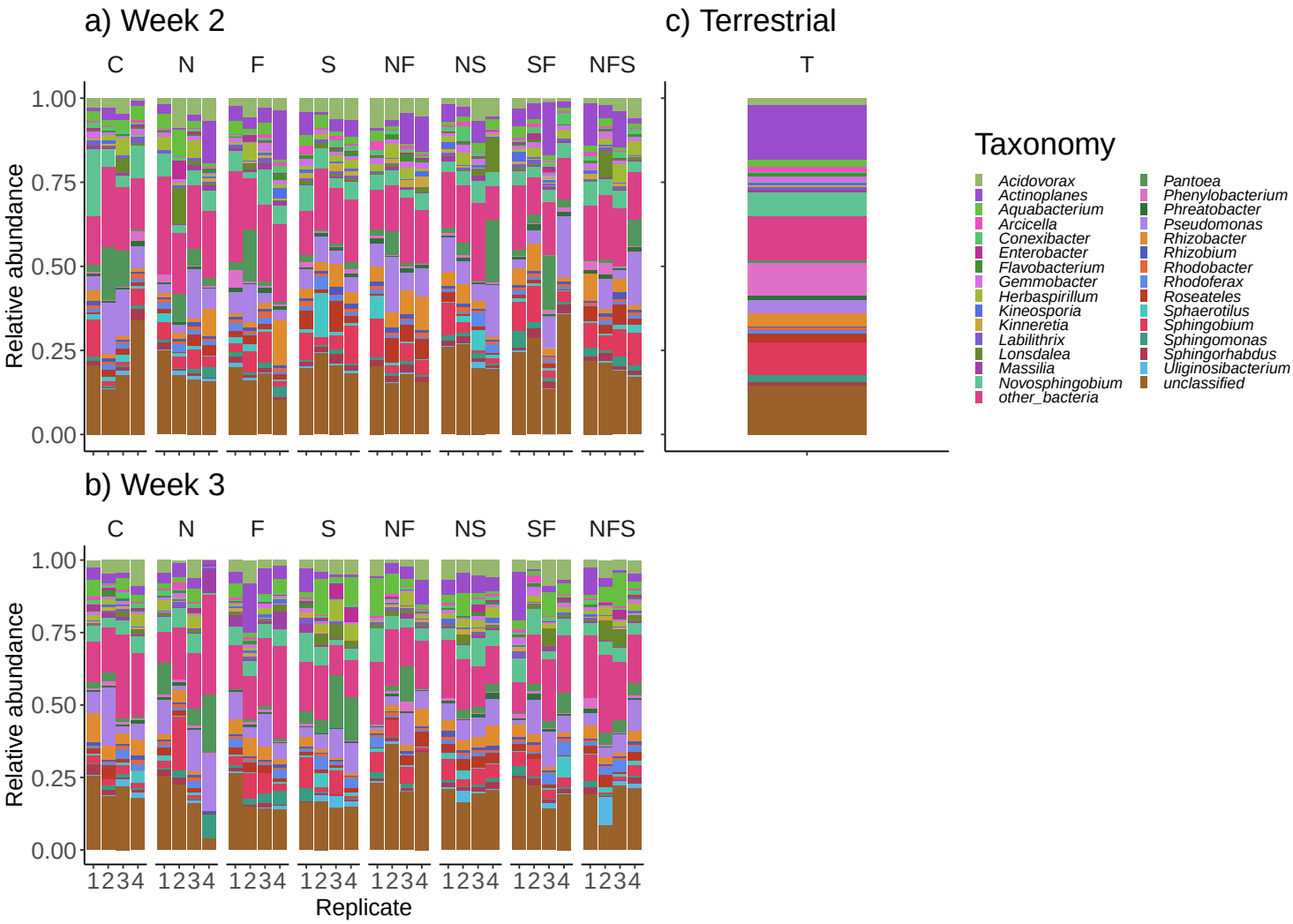


Figure S2.2: Bacterial genera relative abundances in decomposing leaf material after senescence, 2 weeks and 3 weeks of stressor exposure. C: Control; N: Nutrient enrichment; F: Flow velocity reduction; S: Sediment addition; NF: Nutrient + Flow; NS: Nutrient + Sediment; SF: Sediment + Flow; NFS: All three stressors; T: Terrestrial litter

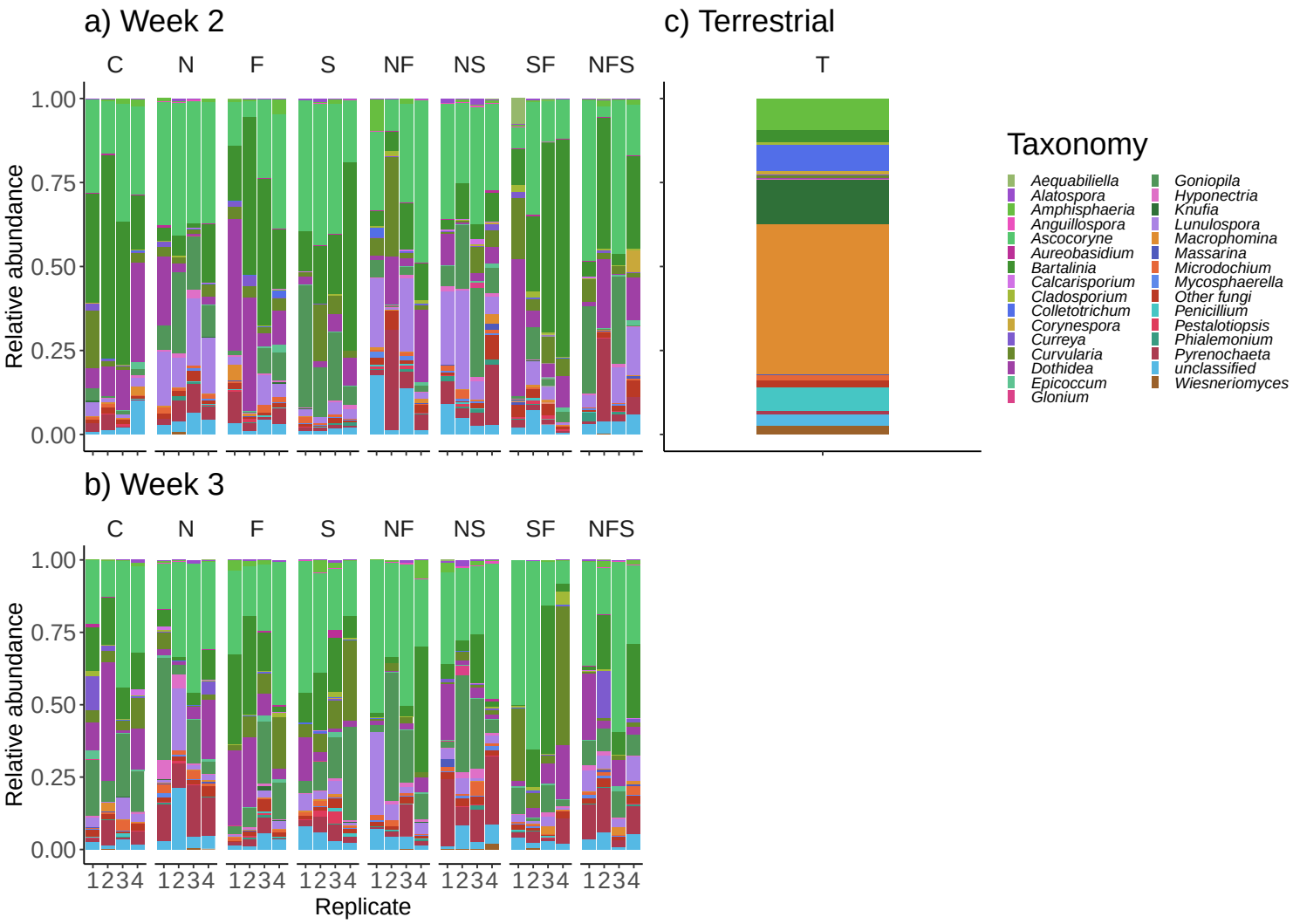


Figure S2.3: Fungal genera relative abundances in decomposing leaf material after senescence, 2 weeks and 3 weeks of stressor exposure. C: Control; N: Nutrient enrichment; F: Flow velocity reduction; S: Sediment addition; NF: Nutrient + Flow; NS: Nutrient + Sediment; SF: Sediment + Flow; NFS: All three stressors; T: Terrestrial litter

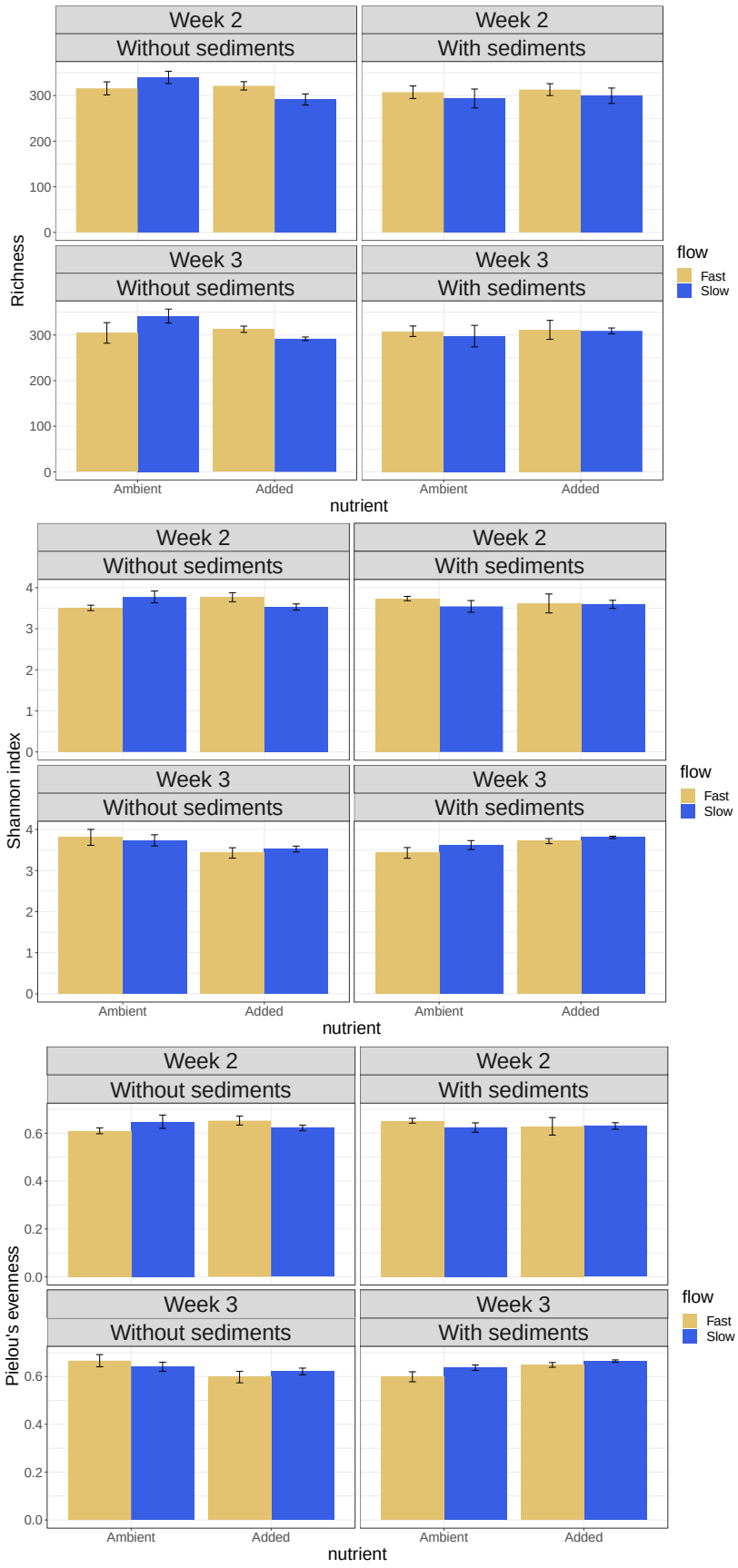


Figure S2.4: Bacterial community genus richness in decomposing leaf material, Shannon's diversity index and Pielou's evenness index (with standard errors) in the experimental treatments.

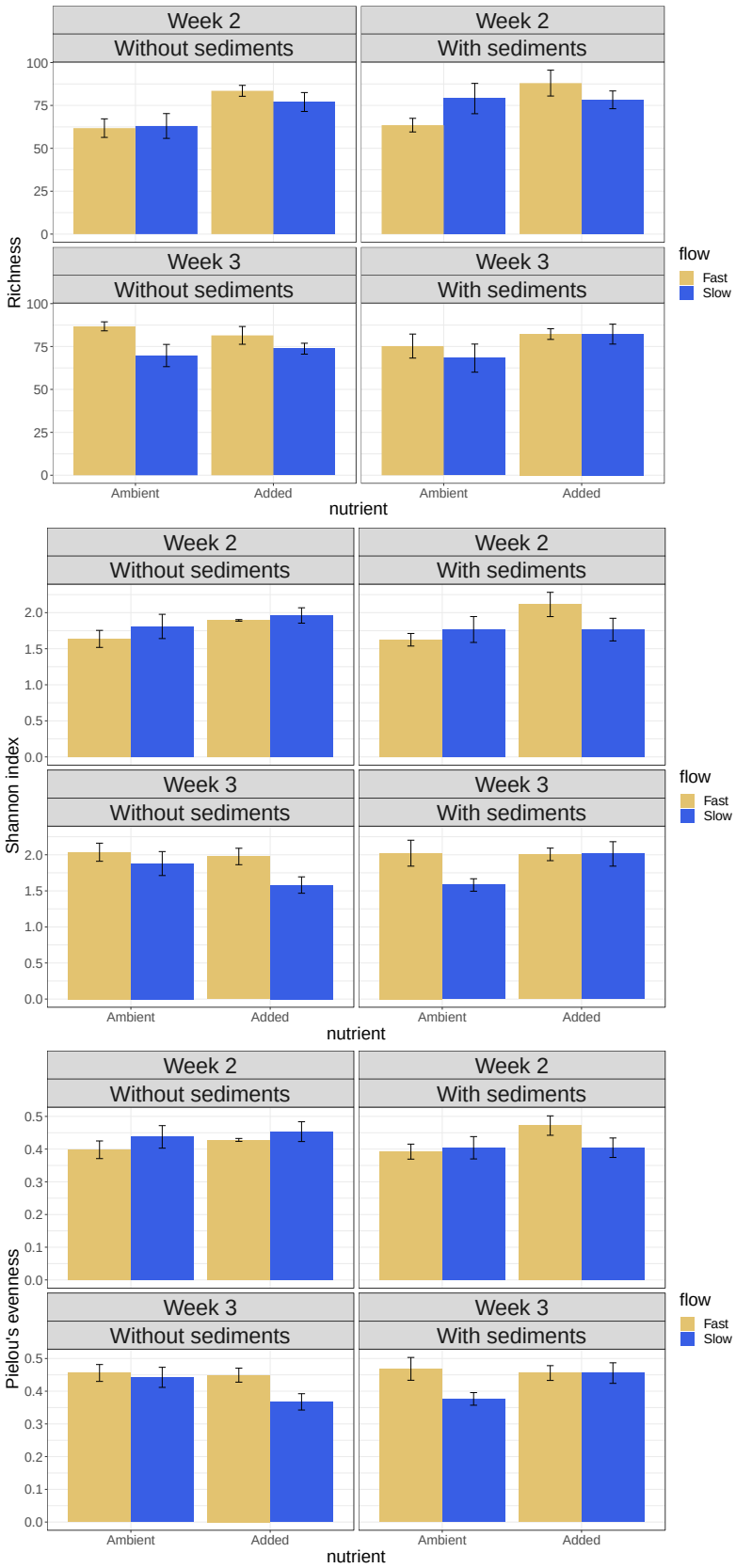


Figure S2.5: Fungal community genus richness in decomposing leaf material, Shannon's diversity index and Pielou's evenness index (with standard errors) in the experimental treatments.

Table S1: Multivariate PERMANOVA results (p-values, F-values and R_2-values) for the total microbial communities (all genera, including rare ones).

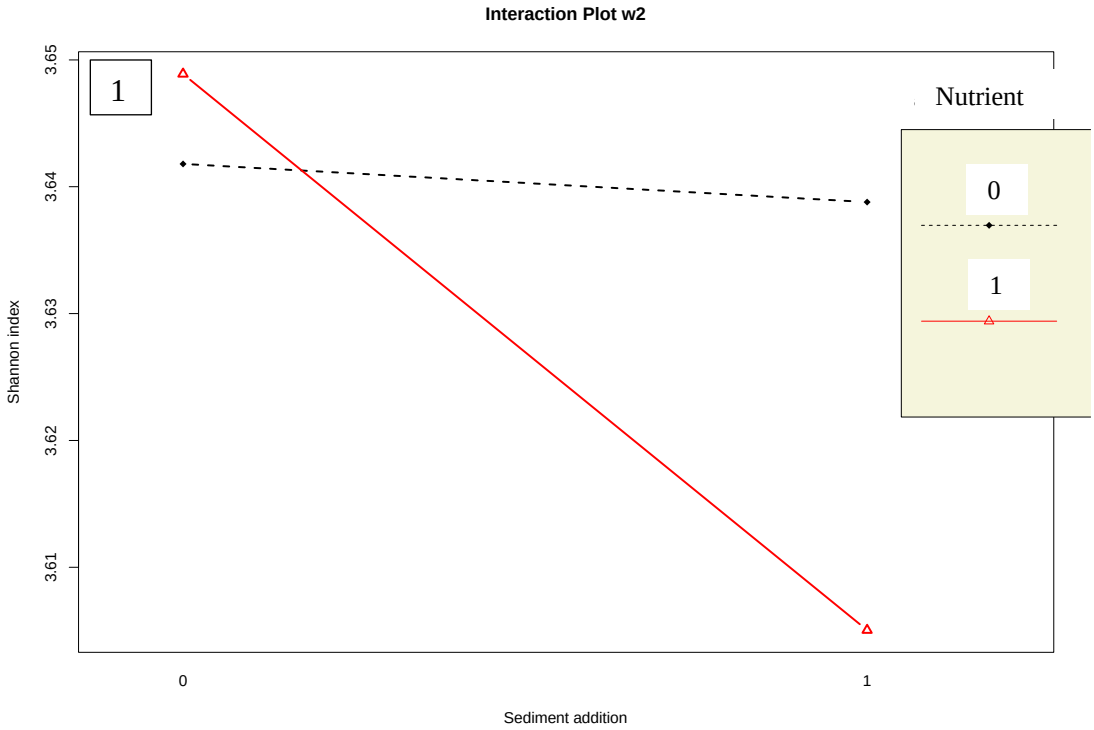
Response	Nutrient	Sediment	Flow	Time	Nutrient X Sediment	Nutrient X Flow	Nutrient X Time	Sediment X Flow	Sediment X Time	Flow X Time	Nutrient X Sediment X Flow	Nutrient X Sediment X Time	Nutrient X Flow X Time	Sediment X Flow X Time	Nutrient X Sediment X Flow X Time
Bacterial community	0.92 F=0.49 R_2=0.007	0.72 F=0.66 R_2=0.01	0.33 F=1.12 R_2= 0.02	0.54 F=1.25 R_2= 0.02	0.82 F=0.60 R_2= 0.01	0.49 F=0.91 R_2=0.01	0.63 F=0.75 R_2=0.01	0.63 F=0.65 R_2=0.01	0.77 F=1.49 R_2=0.02	0.38 F=1.04 R_2=0.02	0.29 F=1.19 R_2=0.02	0.15 F=1.45 R_2=0.02	0.35 F=1.09 R_2=0.02	0.70 F=0.74 R_2=0.01	0.13 F=1.52 R_2=0.02
Fungal community	**0.001** F=8.76, R_2=0.12	0.88 F=0.38, R_2=0.005	**0.04** F=2.45, R_2=0.03	**0.04** F=2.33, R_2= 0.03	0.20 F=1.44, R_2= 0.02	0.98 F=0.15 R_2=0.002	0.70 F=0.61 R_2=0.008	0.15 F=1.60 R_2=0.02	0.70 F=0.61 R_2=0.009	0.17 F=1.50 R_2=0.02	0.85 F=0.44 R_2=0.006	0.99 F=0.06 R_2=0.001	0.46 F=0.88 R_2=0.01	0.83 F=0.44 R_2=0.006	0.66 F=0.66 R_2=0.009

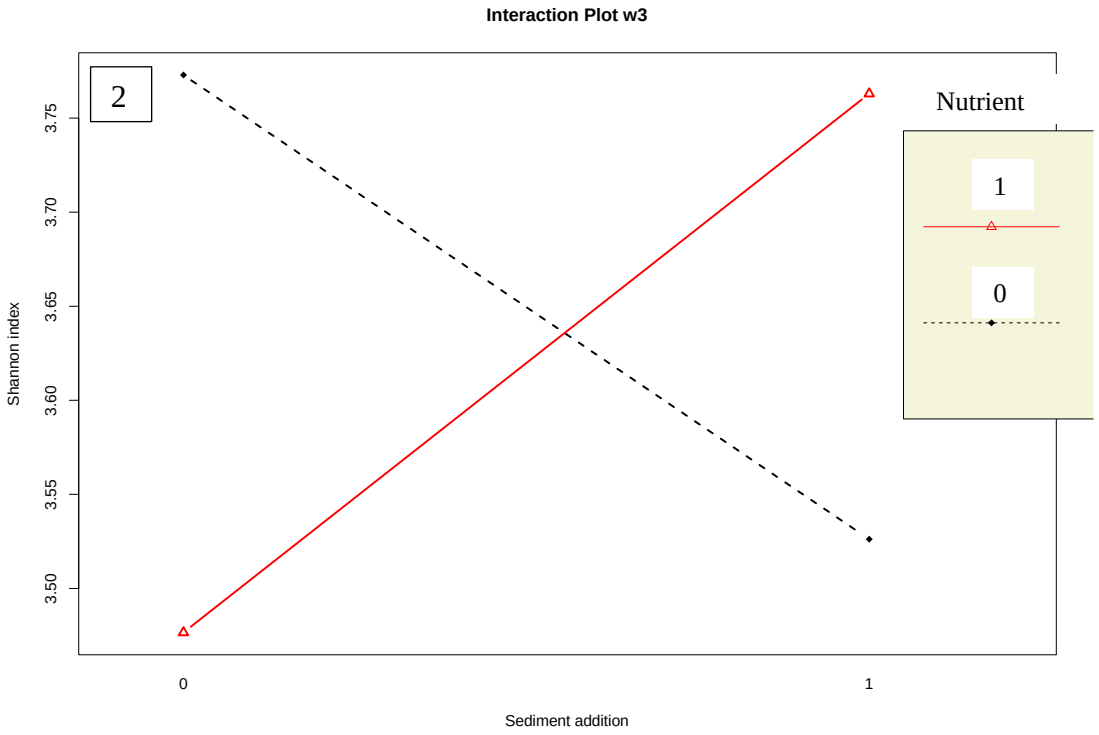
Fig S2.6: Interaction plots and graphs needed for the *Community compositional changes* result section

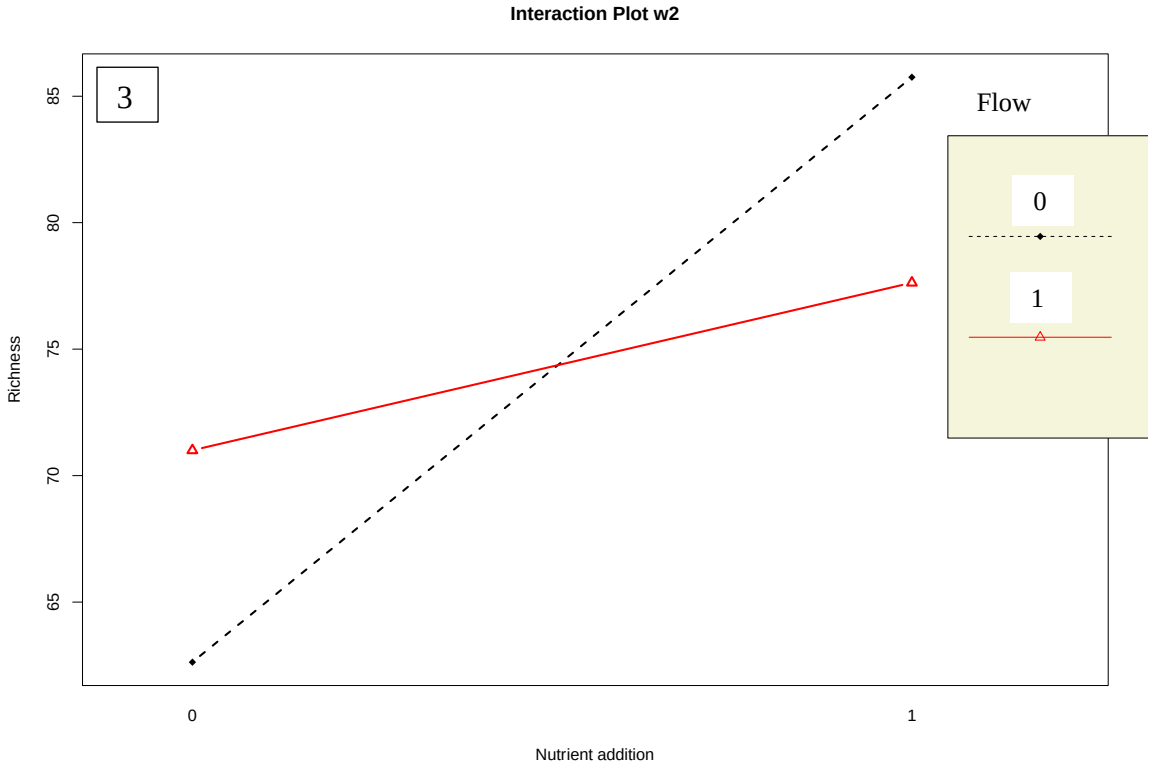
Panels 1- 4, 12 - 21 are the 2-way interaction graphs to assess the direction of the response variable (y-axis) mean values to the different stressor 2-way combinations. The x-axis categorical code 0 and 1 refers to mesocosm without the stressor applied (0) and mesocosm with the stressor applied (1). The same applied for the legend box where the color line associated with 0 refers to mesocosms without the stressors and the line associated with 1 refers to mesocosms where the stressor is applied. The lines represent the direction of the interaction and not a continuous measurement along the x-axis. Therefore, taking panel 1 as an example, when there is no sediment and no nutrient applied to the mesocosm, the mean value of the Shannon diversity is ~3.64. When sediment is added this value decreases slightly below 3.64. When no sediment is added but nutrient concentration is enriched, the mean Shannon diversity of the mesocosms is ~3.65. This value decreases to below 3.61 when both nutrient and sediment are added.

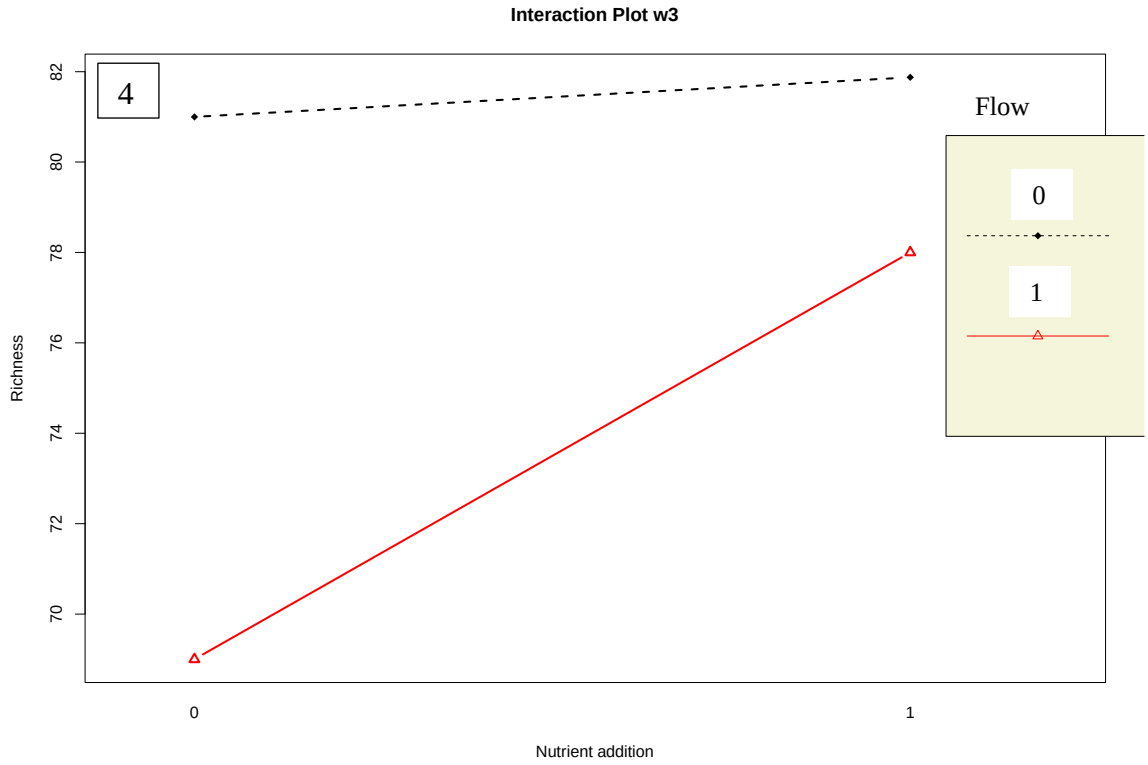
Additional abbreviation used are as follow: w2, Week 2; w3, Week 3.

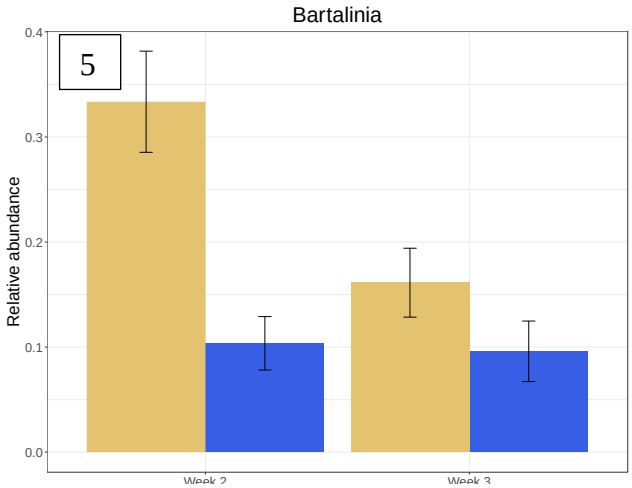
5 – 11 are the bar plots of the response variable mean values with standard errors under different stressor and temporal combinations.

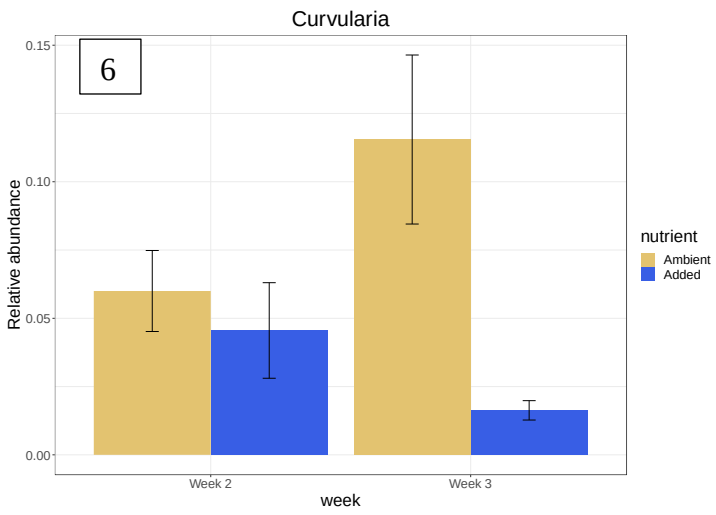


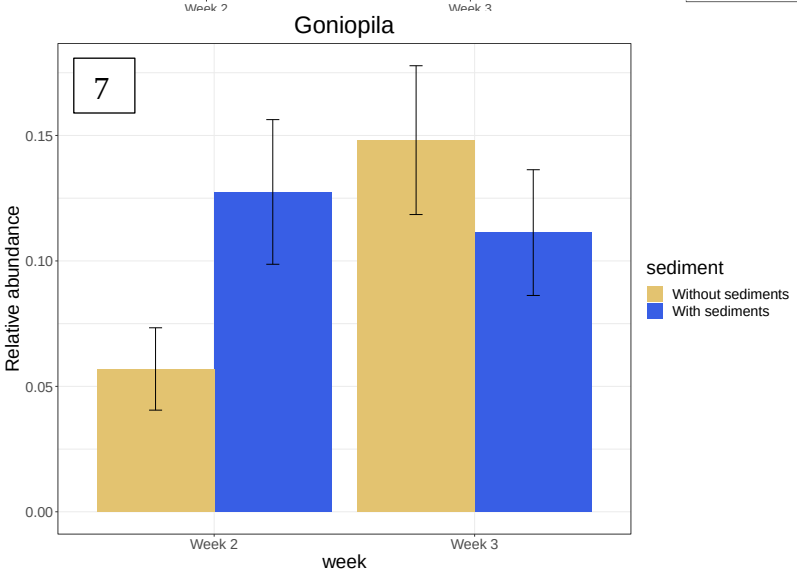

Interaction Plot w3
2
Nutrient
1
0
Shannon index
3.50
3.55
3.60
3.65
3.70
3.75
0
1
Sediment addition

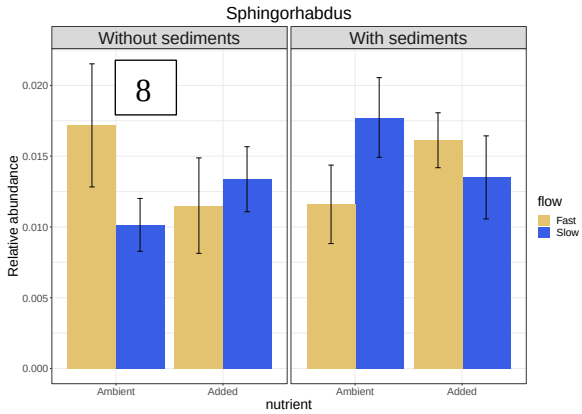

Interaction Plot w2
3
Flow
0
1
Richness
65
70
75
80
85
0
1
Nutrient addition

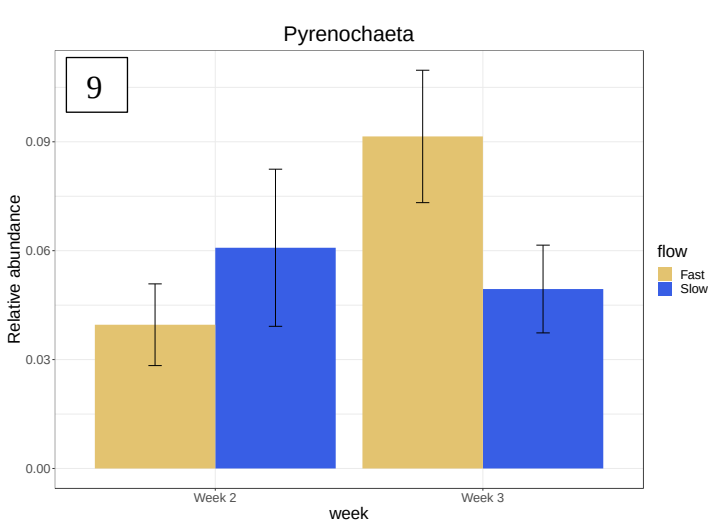

Interaction Plot w3
4
Flow
0
1
Richness
82
80
78
76
74
72
70
0
1
Nutrient addition

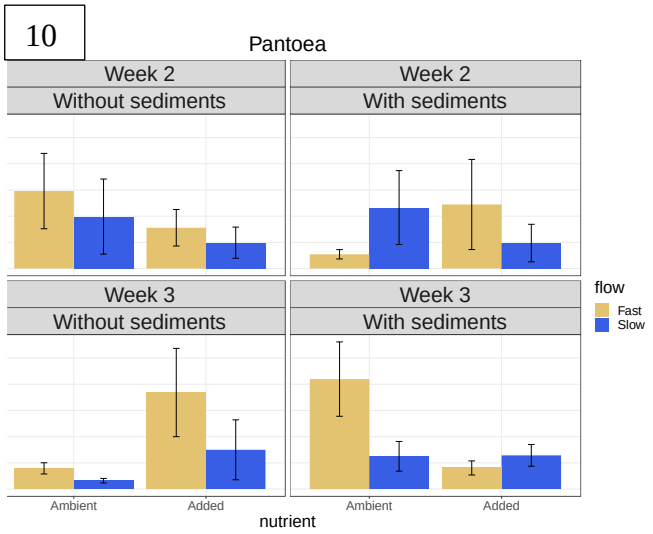

Bartalinia
5
Relative abundance
0.4
0.3
0.2
0.1
0.0
Week 2
Week 3

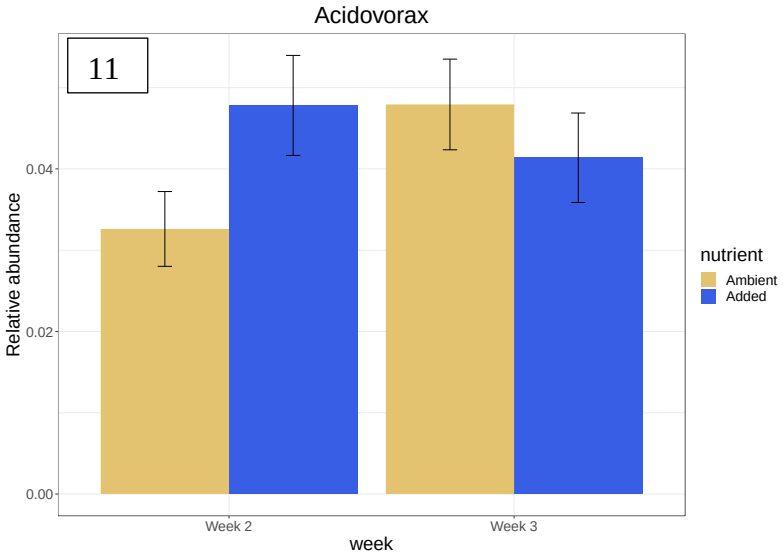

Curvularia
6
Relative abundance
0.15
0.10
0.05
0.00
Week 2
Week 3
week
nutrient
Ambient
Added

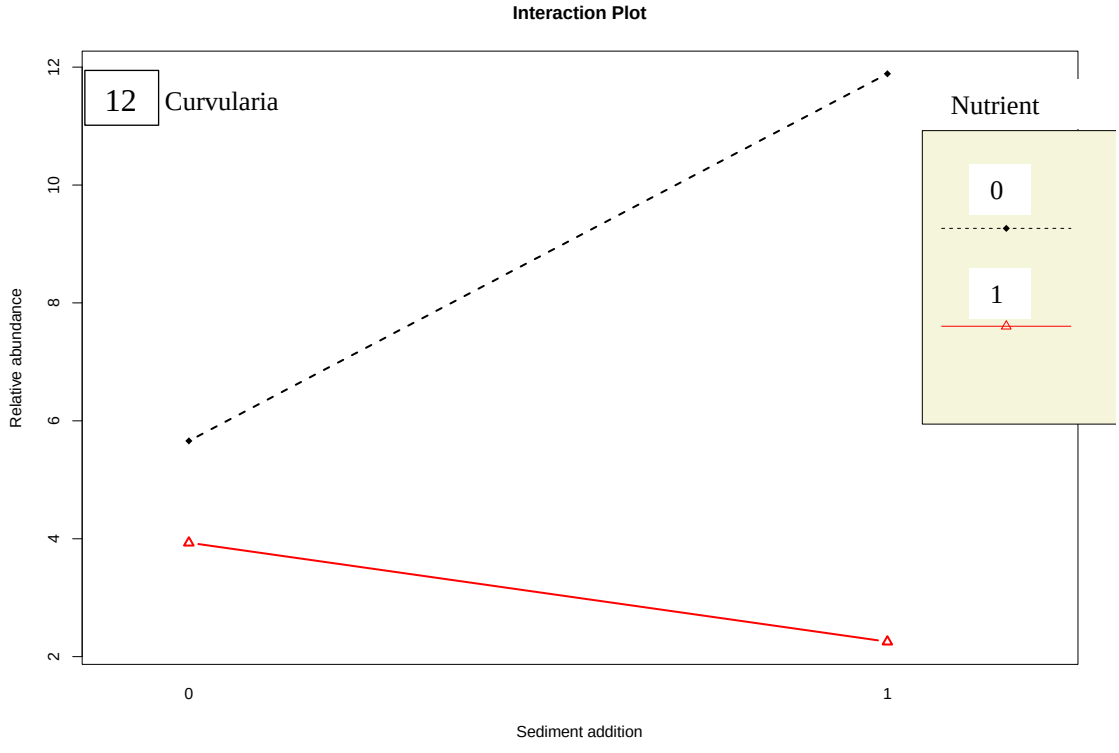

Goniopila
7
Relative abundance
0.15
0.10
0.05
0.00
Week 2
Week 3
week
sediment
Without sediments
With sediments

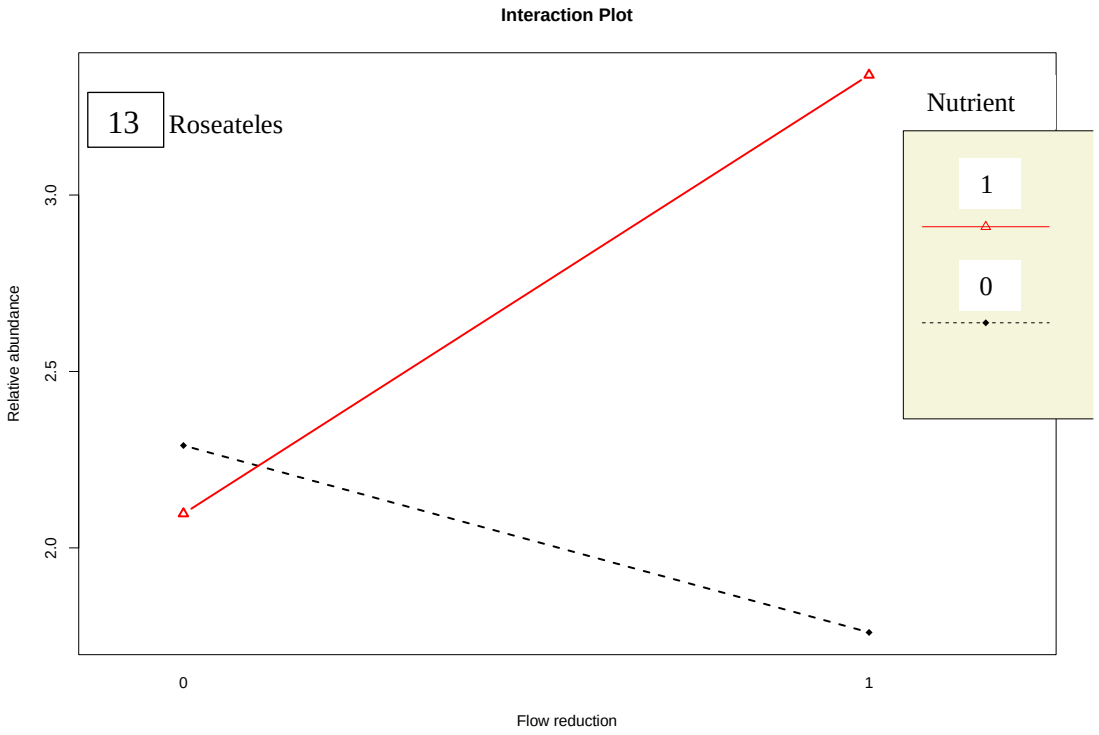

Sphingorhabdus
Without sediments
With sediments
8
Relative abundance
0.020
0.015
0.010
0.005
0.000
Ambient
Added
Ambient
Added
nutrient
flow
Fast
Slow


9
Pyrenochaeta
Relative abundance
0.09
0.06
0.03
0.00
Week 2
Week 3
week
flow
Fast
Slow


10
Pantoea
Week 2
Without sediments
Week 2
With sediments
Week 3
Without sediments
Week 3
With sediments
Ambient
Added
Ambient
Added
nutrient
flow
Fast
Slow


11
Acidovorax
Relative abundance
0.04
0.02
0.00
Week 2
Week 3
week
nutrient
Ambient
Added


Interaction Plot
12
Curvularia
Relative abundance
12
10
8
6
4
2
0
1
Sediment addition
Nutrient
0
1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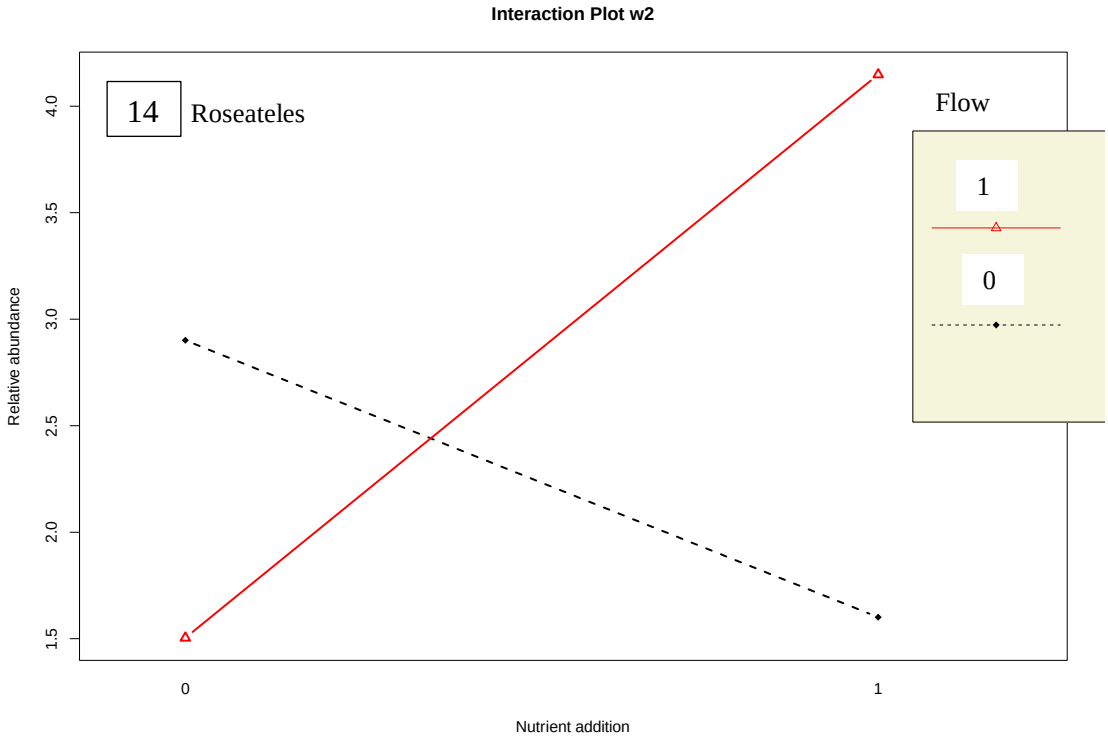
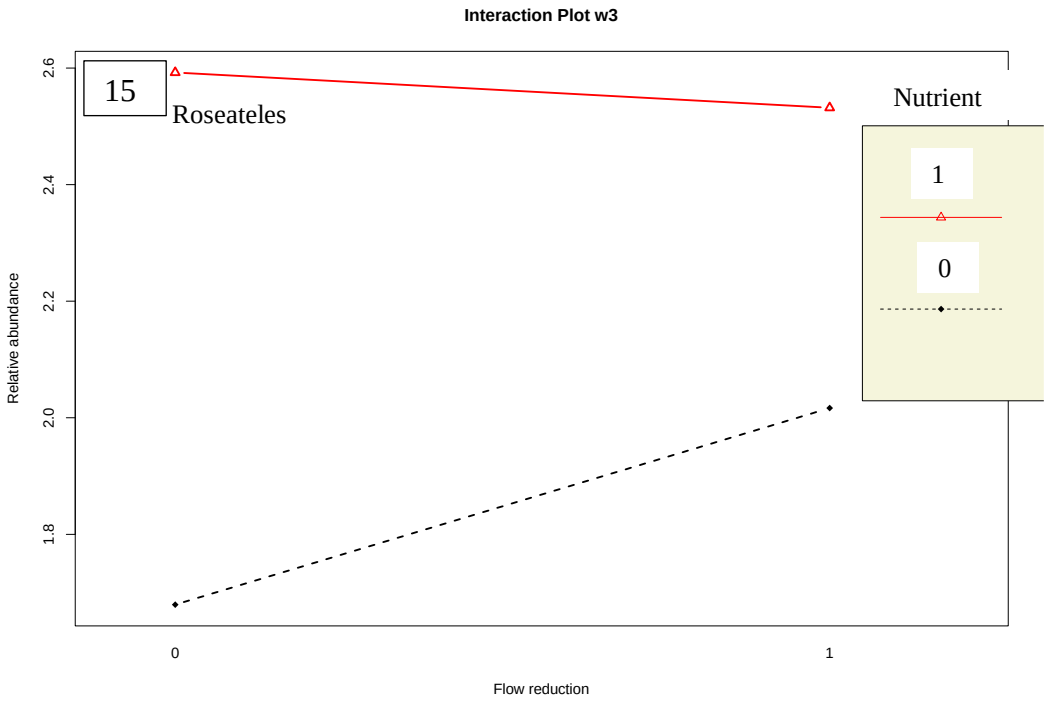
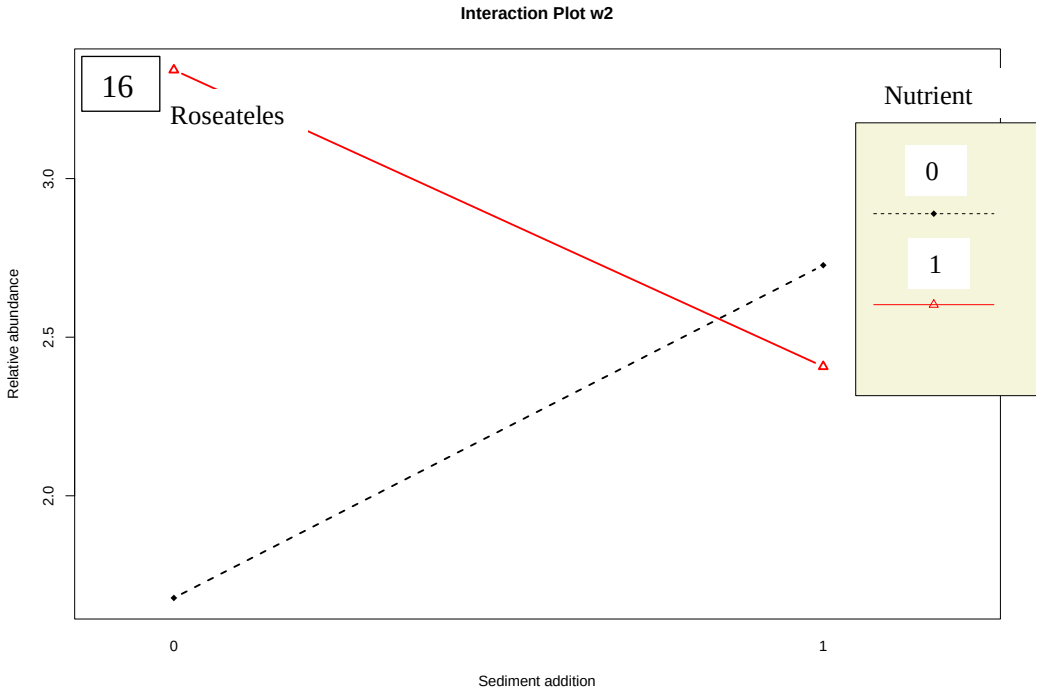
Roseateles
Nutrient
1
0
Relative abundance
3.0
2.5
2.0
0
1
Flow reduction


Interaction Plot w2
14
Roseateles
Flow
1
0
Relative abundance
4.0
3.5
3.0
2.5
2.0
1.5
0
1
Nutrient addition


Interaction Plot w3
15
Roseateles
Nutrient
1
0
Relative abundance
2.6
2.4
2.2
2.0
1.8
0
1
Flow reduction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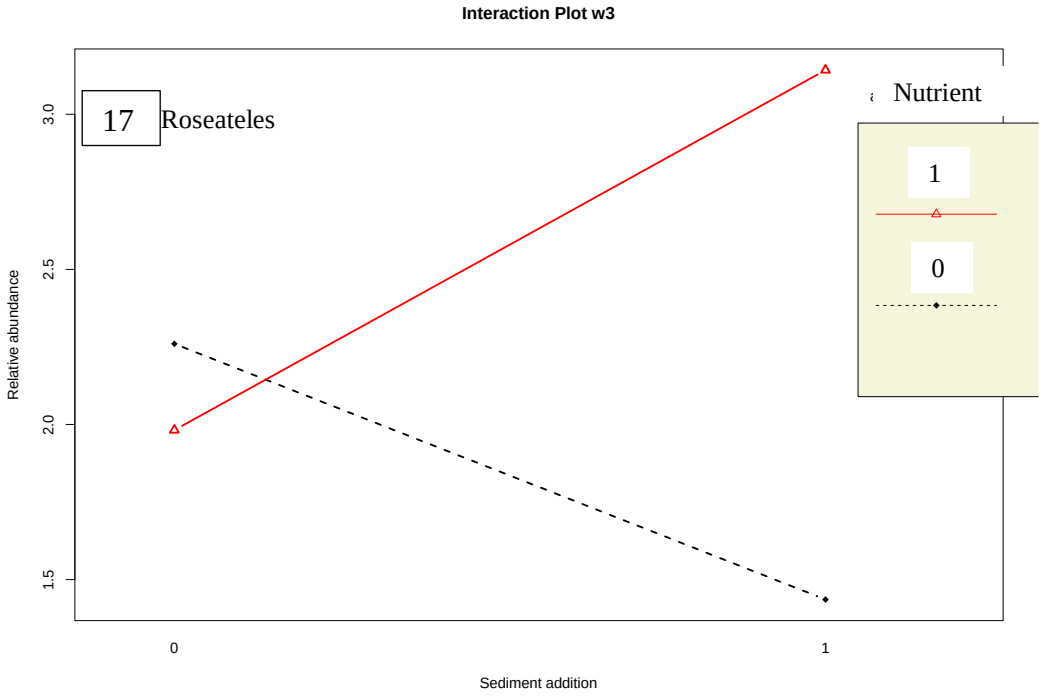
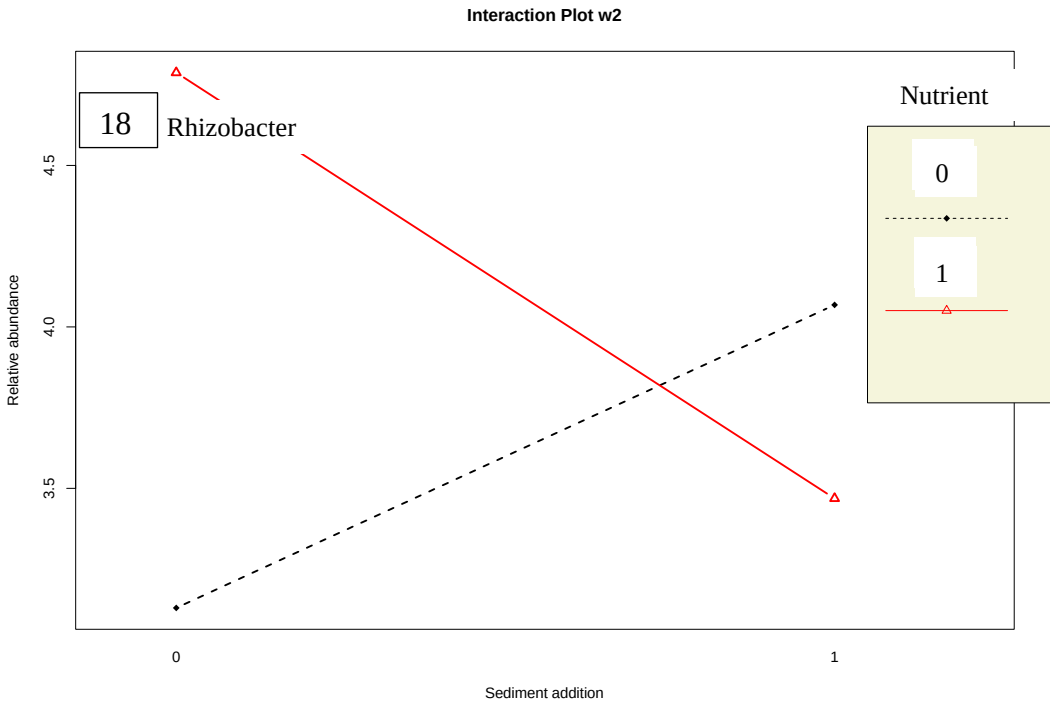
16
Roseateles
Nutrient
0
1
Relative abundance
3.0
2.5
2.0
0
1
Sediment addition


Interaction Plot w3
17
Roseateles
Nutrient
1
0
Relative abundance
3.0
2.5
2.0
1.5
0
1
Sediment addition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Nutrient
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1
Relative abundance
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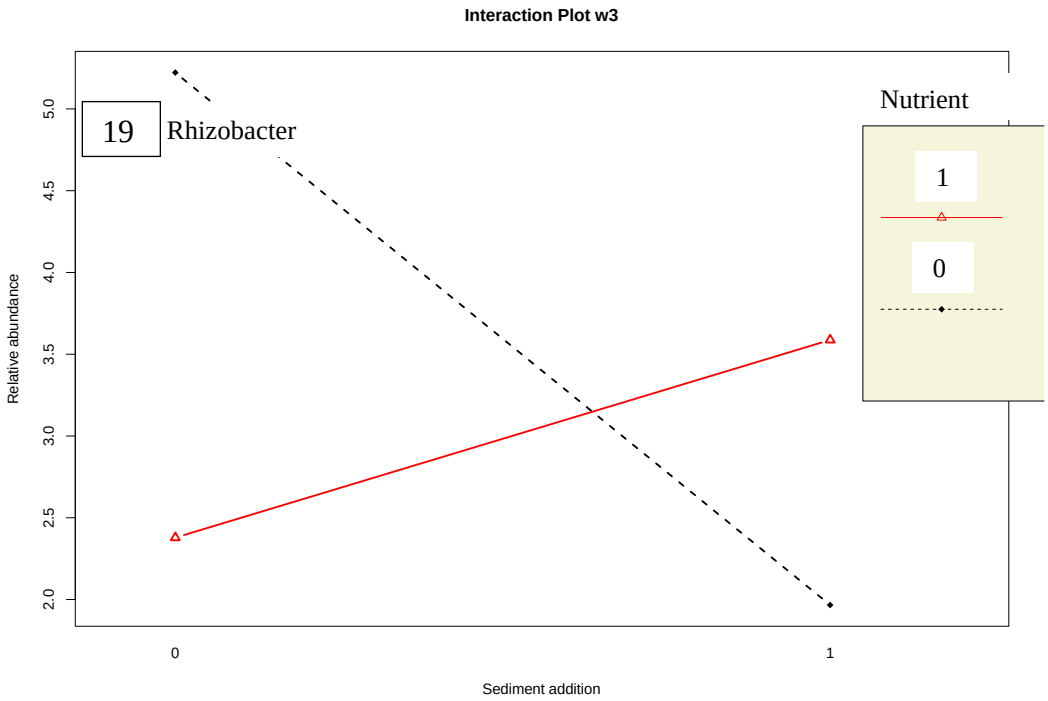
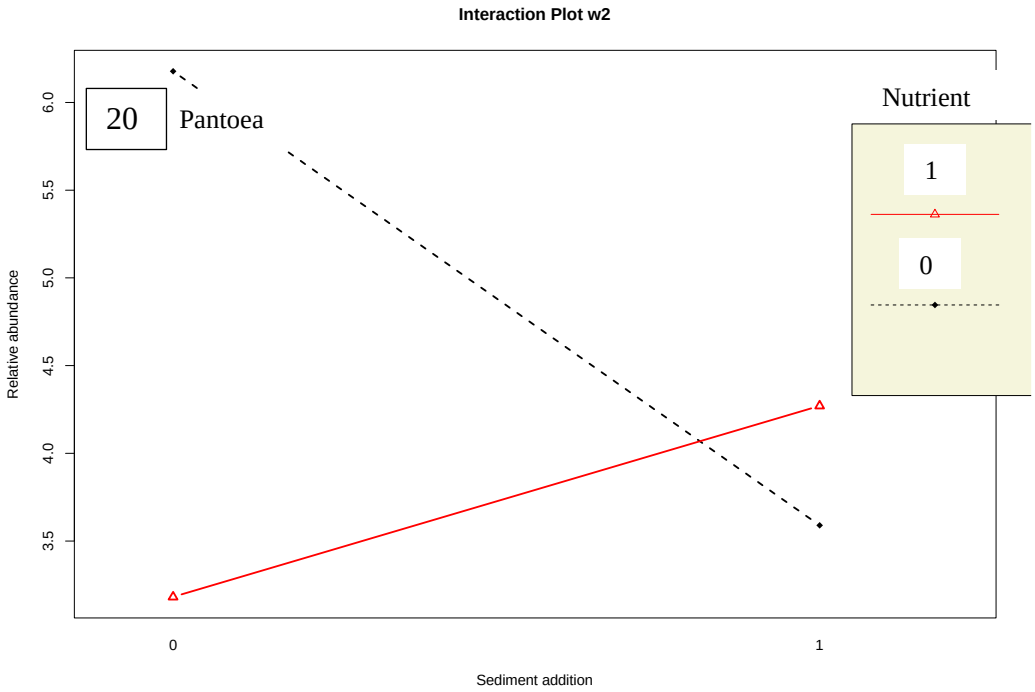
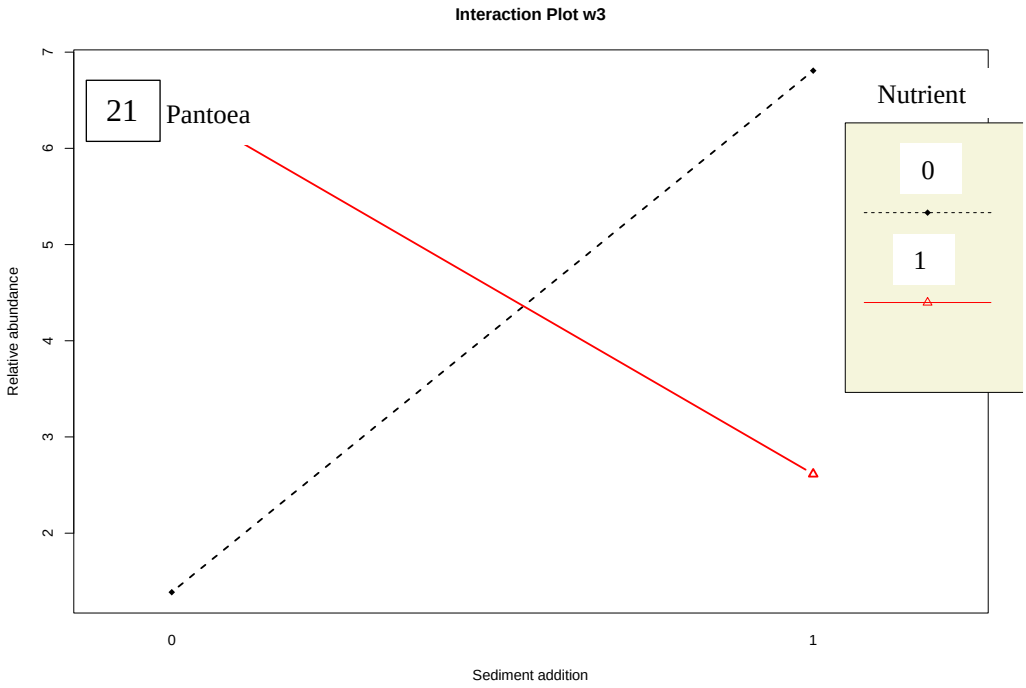
4.0
3.5
0
1
Sediment addition


Interaction Plot w3
19
Rhizobacter
Nutrient
1
0
Relative abundance
2.0
2.5
3.0
3.5
4.0
4.5
5.0
0
1
Sediment addition


Interaction Plot w2
20
Pantoea
Nutrient
1
0
Relative abundance
3.5
4.0
4.5
5.0
5.5
6.0
0
1
Sediment addition


Interaction Plot w3
21
Pantoea
Nutrient
0
1
Relative abundance
2
3
4
5
6
7
0
1
Sediment addition

Detailed description of the interaction patterns for the abundant bacterial general:

Interactions among the stressors affected all but one (*Rhizobiales*) of the 13 abundant taxa. Nutrients x sediment interactions occurred in seven taxa (53.8%). Relative abundances of *Planctomycetales* (Fig. S3.13), *Spartobacteria_uncl* (Fig. S3.21), *Sphingobacteriales* (Fig. S3.23) and *Xanthomonadales* (Fig. S3.30) all increased more strongly in nutrient-enriched than in non-enriched mesocosms when sediment was added. The opposite was seen in *Pseudomonadales* (faster decrease with sediment in nutrient-enriched mesocosms, Fig. S3.16). *Sphingomonadales* barely responded to sediment addition alone but decreased markedly when both sediment and nutrients were added (Fig. S3.25). Sediment alone decreased *Verrucomicrobiales*, but this effect was reversed when nutrients were also added (Fig. S3.27).

Nutrients x flow velocity interactions occurred in two taxa (15.4%). Both *Acidimicrobiales* (Fig. S3.7) and *Rhodabacterales* (Fig. S3.18) decreased in relative abundance at fast flow velocity when nutrients were added but this response was reversed at reduced flow velocity.

Sediment x flow velocity interactions occurred in 12 taxa (92.3%). *Acidimicrobiales* (Fig. S3.8, this interaction overrode the positive flow velocity reduction main effect) and *Actinomycetales* (Fig. S3.10) both increased more strongly with sediment addition in fast-flowing mesocosms. *Burkhodariales* (Fig. S3.11), *Rhodobacterales* (Fig. S3.19), *Rhodospirillales* (Fig. S3.20), *Verrucomicrobiales* (Fig. S3.28) and *Xanthomonadales* (Fig. S3.29) all increased when sediment alone was added but either lost this increase (*Burkhodariales*, *Xanthomonadales*; this interaction overrode the positive sediment main effect on these taxa) or decreased when added sediment was combined with flow velocity reduction. The opposite pattern (negative response to sediment lost when combined with flow velocity reduction) was observed in *Pseudomonadales* (Fig. S3.14) and *Sphingomonadales* (Fig. S3.26; this interaction overrode the negative sediment main effect for both taxa). *Planctomycetales* (Fig. S12), *Spartobacteria_uncl* (Fig. S3.22) and *Sphingobacteriales* (Fig. S3.24) all increased with added sediment at fast flow velocity, but less so (or not at all: *Planctomycetales*) at reduced flow velocity.

Three-way interactions among all stressors occurred for four taxa (30.7%). All were relatively weak and did not override any lower-order interactions. *Acidimicrobiales* (Fig. S3.9), *Rhodobacterales* (Fig. S3.17) and *Xanthomonadales* (Fig. S3.31) all increased in relative abundance when flow velocity was reduced, especially in nutrient-enriched mesocosms, but this positive effect was diminished or disappeared in the presence of sediment (were relative abundances were similarly high at both flow velocities). By contrast, *Pseudomonadales* were most abundant in mesocosms without sediment and enriched in nutrients, but decreased strongly when flow velocity was reduced (Fig. S3.15). This negative effect was dampened in mesocosms with added sediment where the taxon was generally much less abundant.

Detailed description of the interaction patterns for the abundant function:

Stressor interactions were common, with four (57.1%) of the seven abundant bacterial functions showing nutrients x sediment, six (85.7%) sediment x flow velocity and three (42.8%)

nutrients x sediment x flow velocity interactions. Sulfide oxidizers (Fig. S3.36) and xylan degraders (Fig. S3.43) increased more strongly when sediment and nutrients were added together than when only sediment was added. Sulfate reducers (Fig. S3.47) and ammonia oxidizers (Fig. S3.49) showed the opposite pattern (stronger decrease when both sediment and nutrients added than for sediment alone).

Sulfide oxidizers (Fig. S3.35) & chitin degraders (Fig. S3.39) increased strongly with sediment addition at fast flow but much less so at reduced flow velocity. This interaction overrode the weaker flow velocity main effect on the two taxa. Xylan degraders (Fig. S3.42) and nitrogen fixers (Fig. S3.45) increased more markedly with sediment addition at fast than at reduced flow velocity. Sulfate reducers (Fig. S3.46) and ammonia oxidizers (Fig. S3.48) showed the opposite pattern (stronger decrease with sediment addition at fast than at reduced flow velocity).

Three-way interactions occurred for three of the seven bacterial functions (42.8%). All were weak and did not override any lower-order interactions. Chitin degraders (Fig. S3.38), xylan degraders (Fig. S3.41) and nitrogen fixers (Fig. S3.44) all increased in relative abundance when flow velocity was reduced, especially in nutrient-enriched mesocosms, but this positive effect was lessened or disappeared in the presence of sediment (where relative abundances were similarly high at both flow velocities).

Finally, two stressor main effects changed with time for sulfide oxidizers (flow velocity x time: positive effect of velocity reduction stronger after 3 weeks than after 2 weeks, Fig. S3.33; sediment x time: positive effect of added sediment weaker after 3 weeks, Fig. S3.34), and so did the shape of the 3-way interaction for three functions (chitin degraders, dehalogenizers, sulfide oxidizers). The latter two interactions occurred for functions where the three-way interaction itself was not significant, therefore these 4-way interactions will not be interpreted because they are unlikely to be biologically meaningful. For chitin degraders, in week 2 abundances in mesocosms with sediment addition were lower at reduced flow velocity than at fast flow in both nutrient treatments, but in week 3 this flow-velocity-related pattern was reversed in non-enriched mesocosms with added sediment (Fig. S3.37).

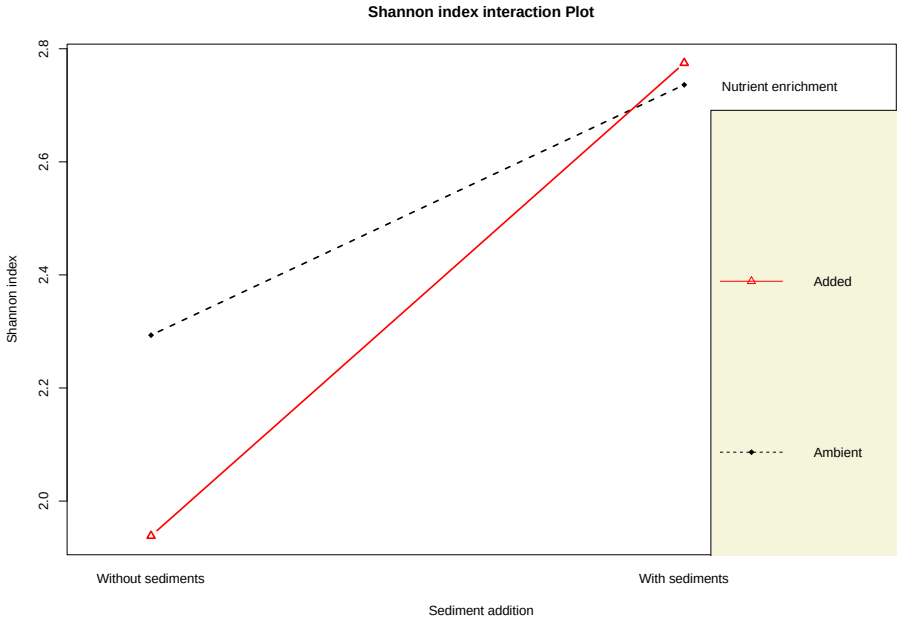


Figure S3.1: Interaction plot between the sediment and nutrient addition treatments for the Shannon diversity index.

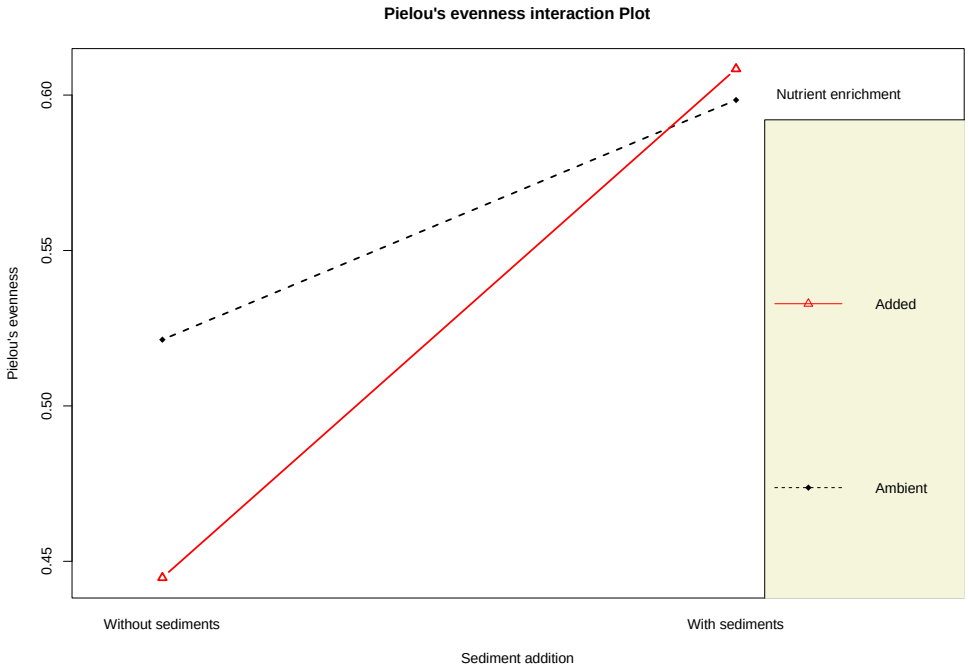


Figure S3.2: Interaction plot between the sediment and nutrient addition treatments for Pielou's evenness index.

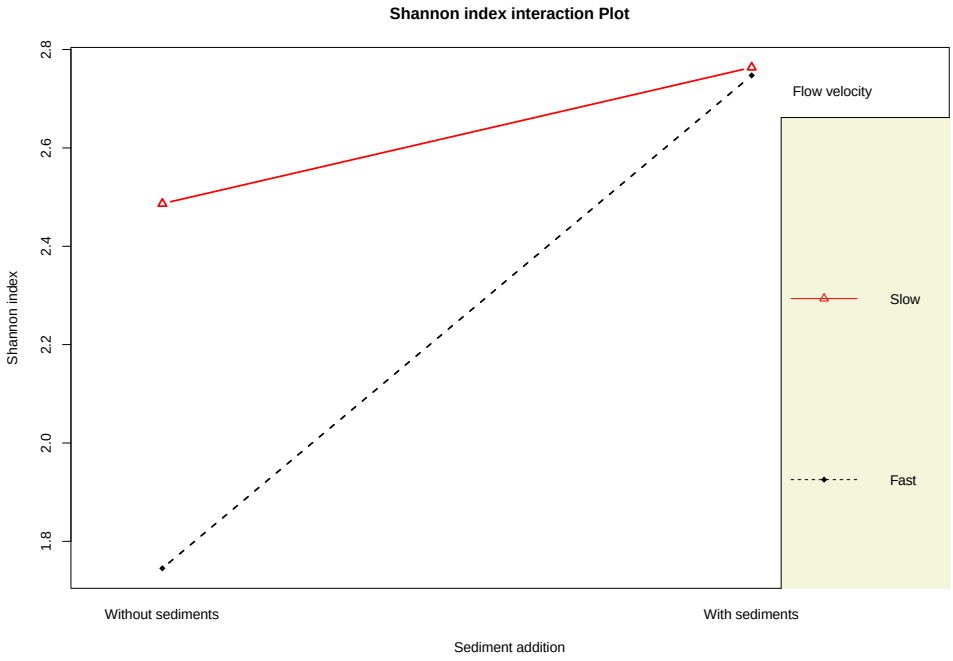


Figure S3.3: Interaction plot between the sediment and flow velocity reduction addition treatments for the Shannon diversity index.

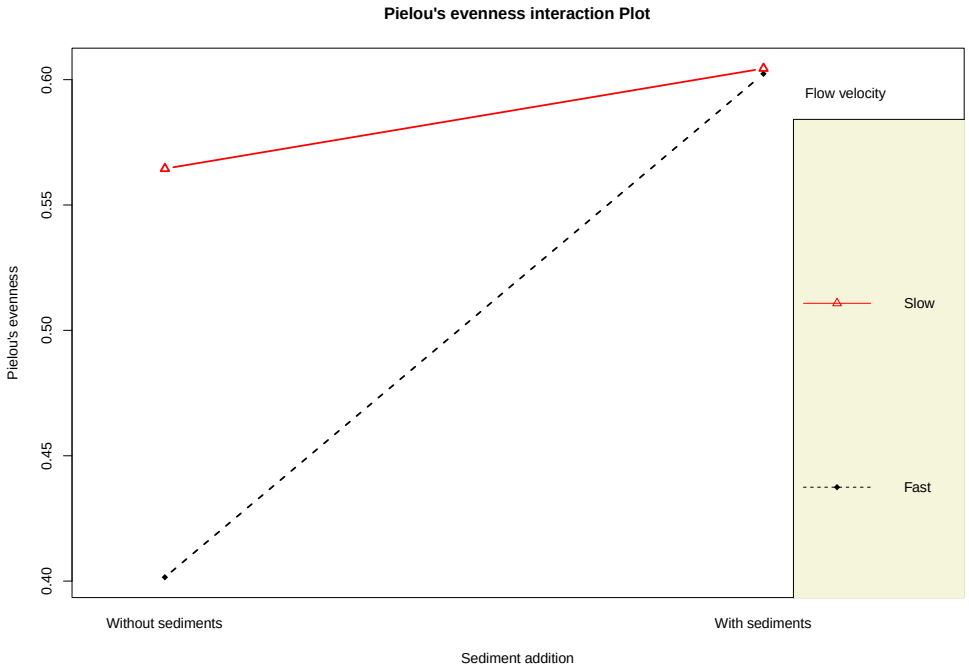


Figure S3.4: Interaction plot between the sediment and flow velocity reduction treatments for Pielou's evenness index.

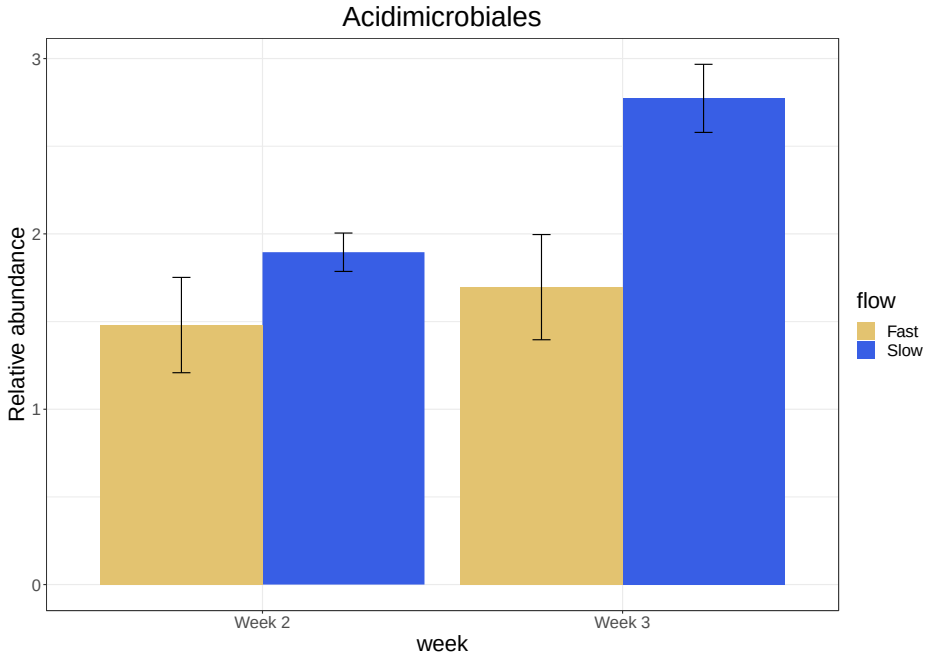


Figure S3.5: Relative abundance of Acidimicrobiales (mean +- SE) in the flow velocity treatments across the two sampling dates.

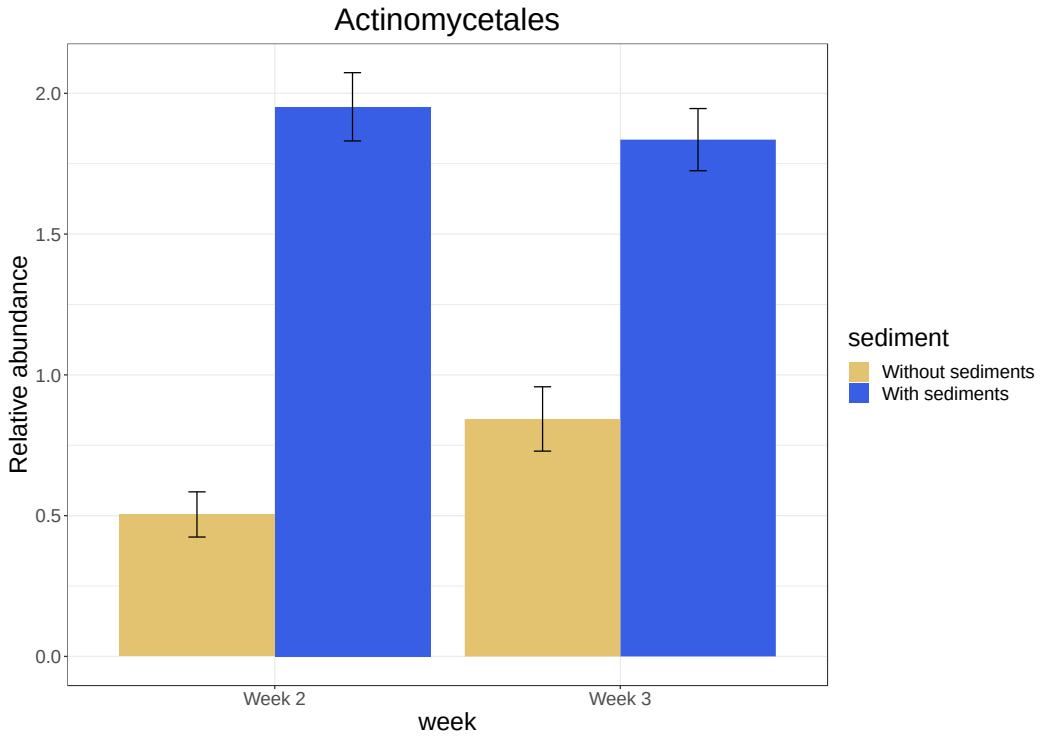


Figure S3.6: Actinomycetales relative abundance evolution through time of exposure to the sediment treatment.

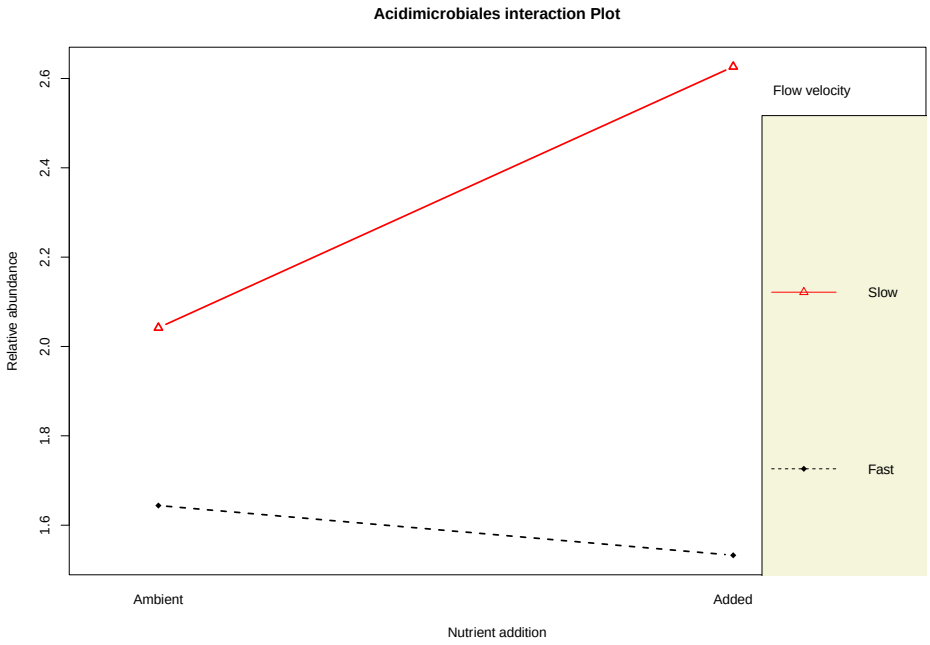


Figure S3.7: Relative abundance of Acidimicrobiales: interaction plot between the flow velocity reduction and nutrient treatments.

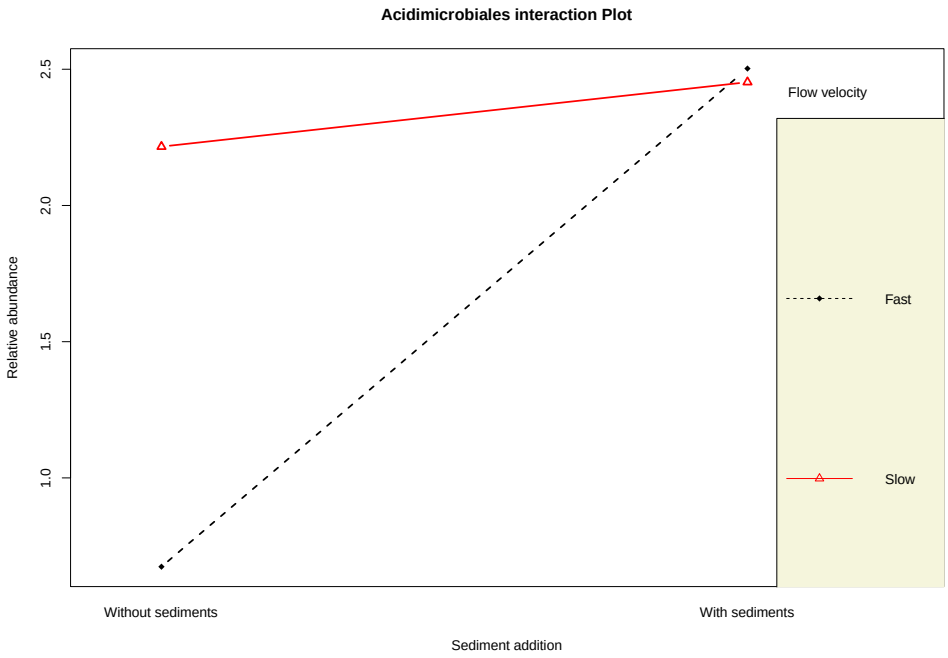


Figure S3.8: Acidimicrobiales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

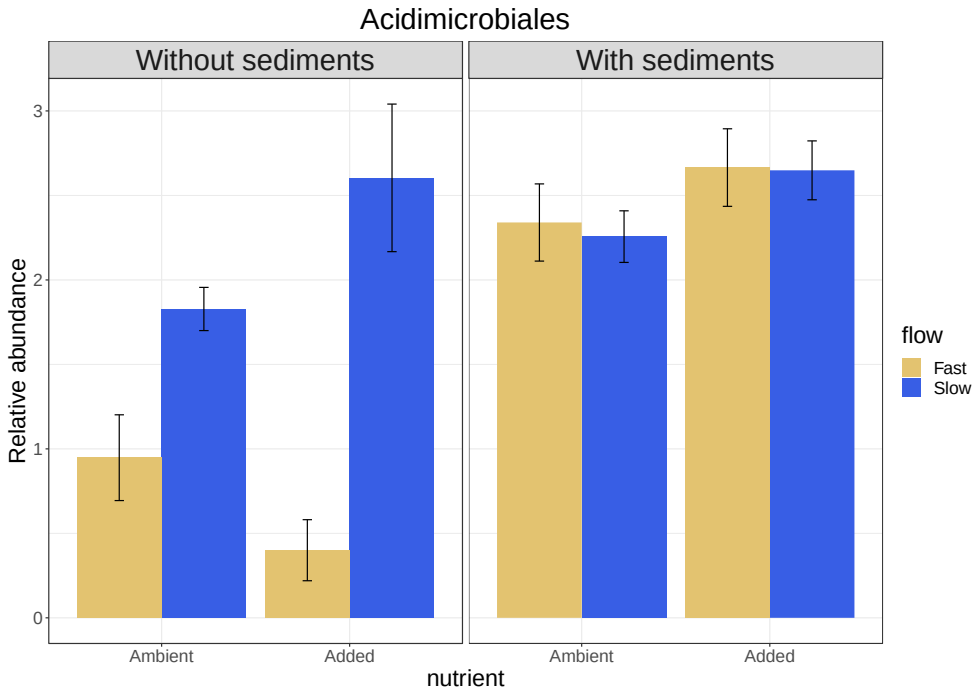


Figure S3.9: Acidimicrobiales relative abundance interaction plot between the three stressors.

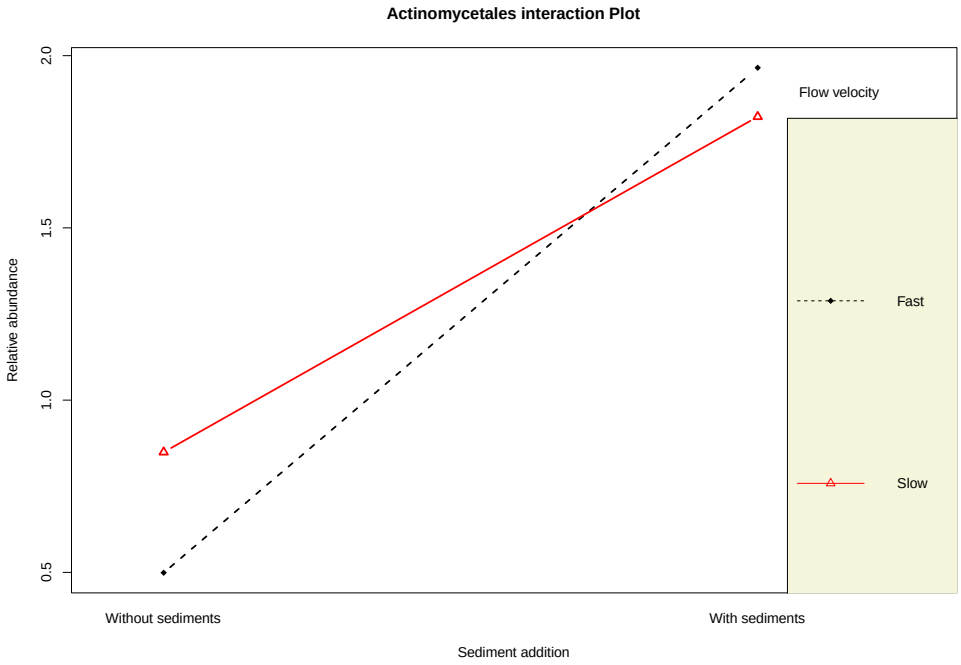


Figure S3.10: Actinomycetales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

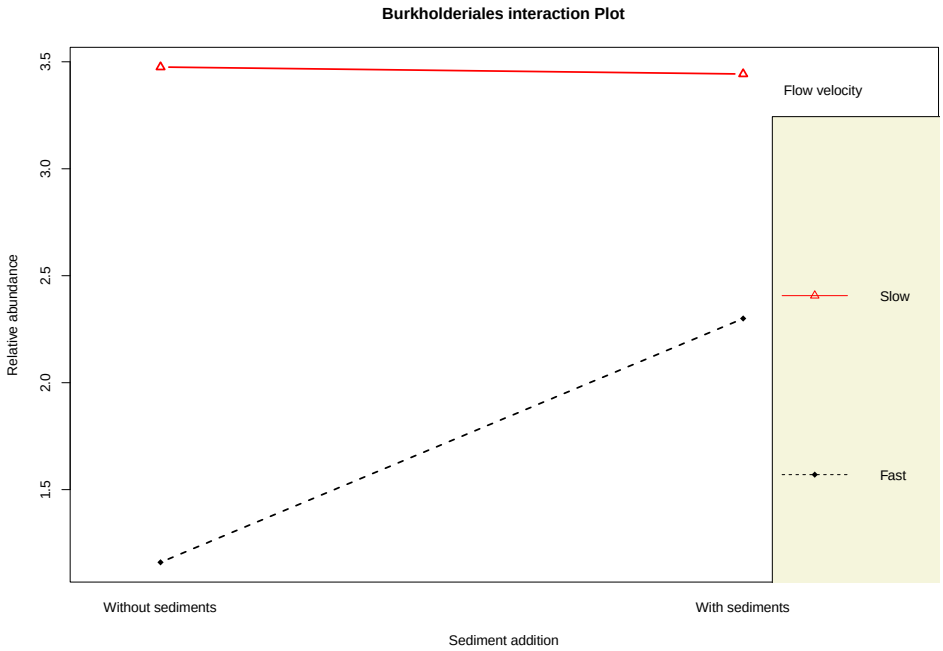


Figure S3.11: Burkholderiales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

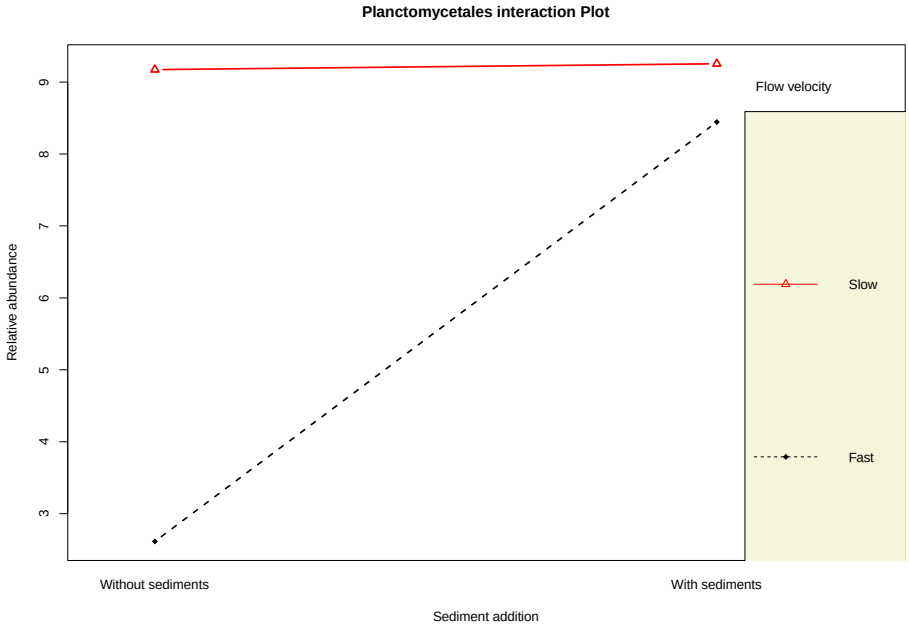


Figure S3.12: Planctomycetales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

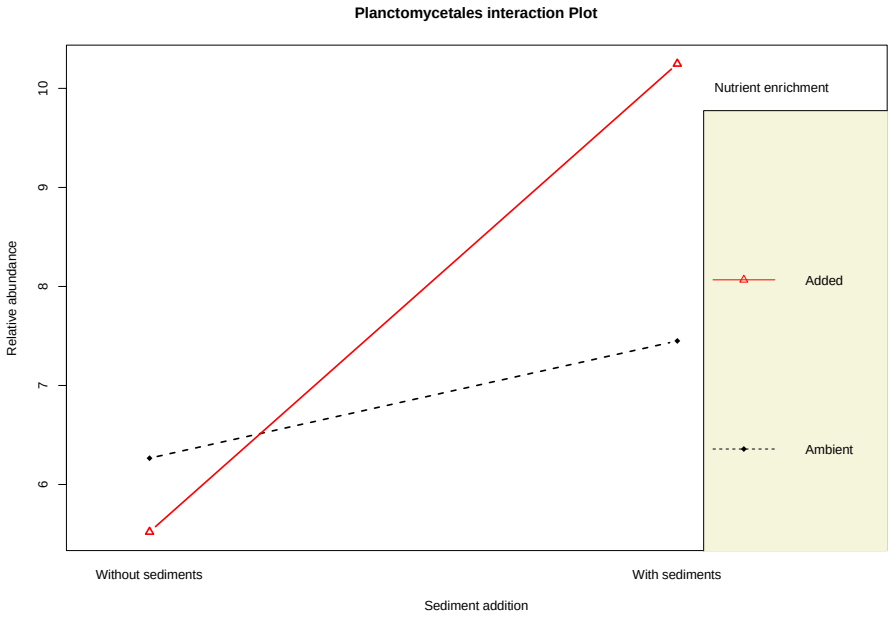


Figure S3.13: Planctomycetales relative abundance interaction plot between the sediment and nutrient treatment.

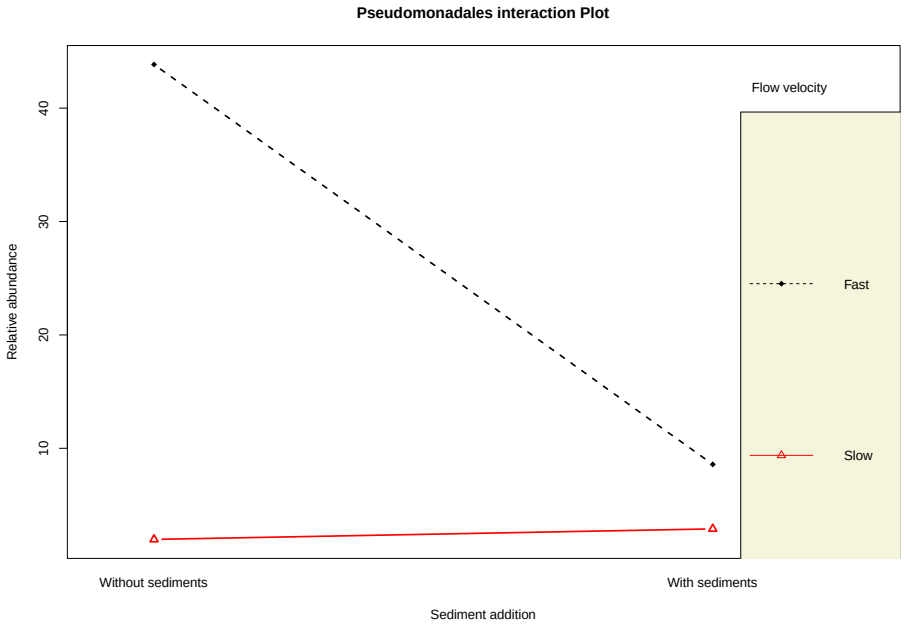


Figure S3.14: Pseudomonadales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

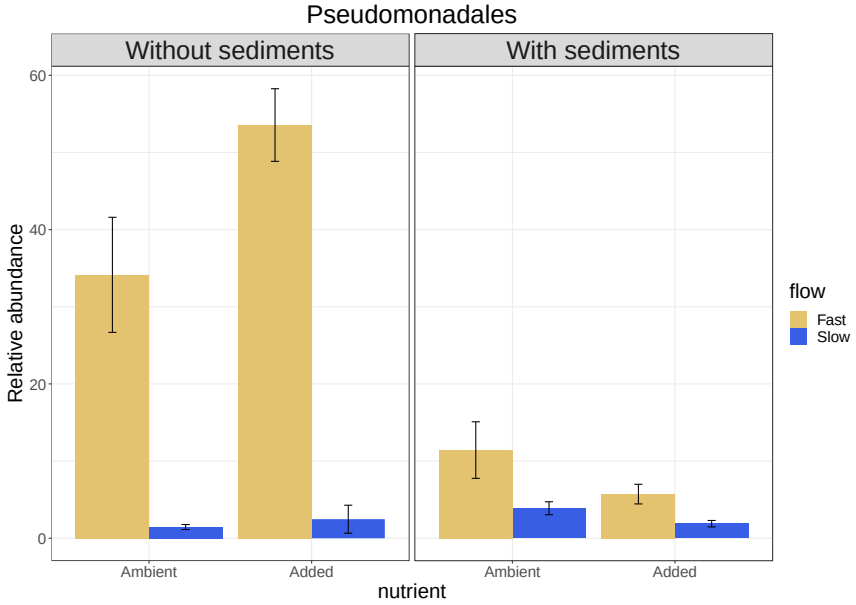


Figure S3.15: Pseudomonadales relative abundance interaction plot between the three stressors.

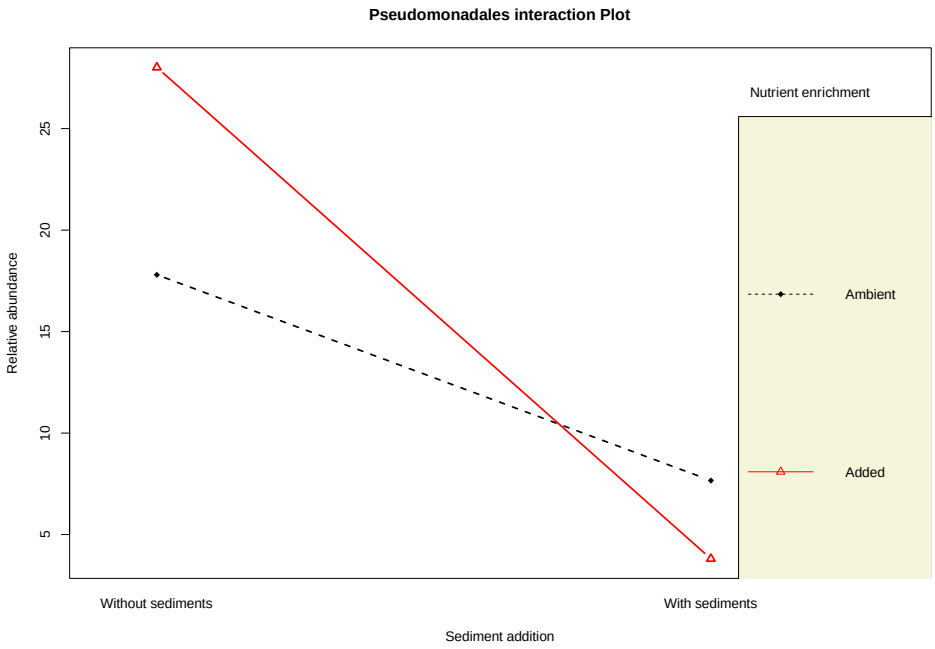


Figure S3.16: Pseudomonadales relative abundance interaction plot between the sediment and nutrient treatment.

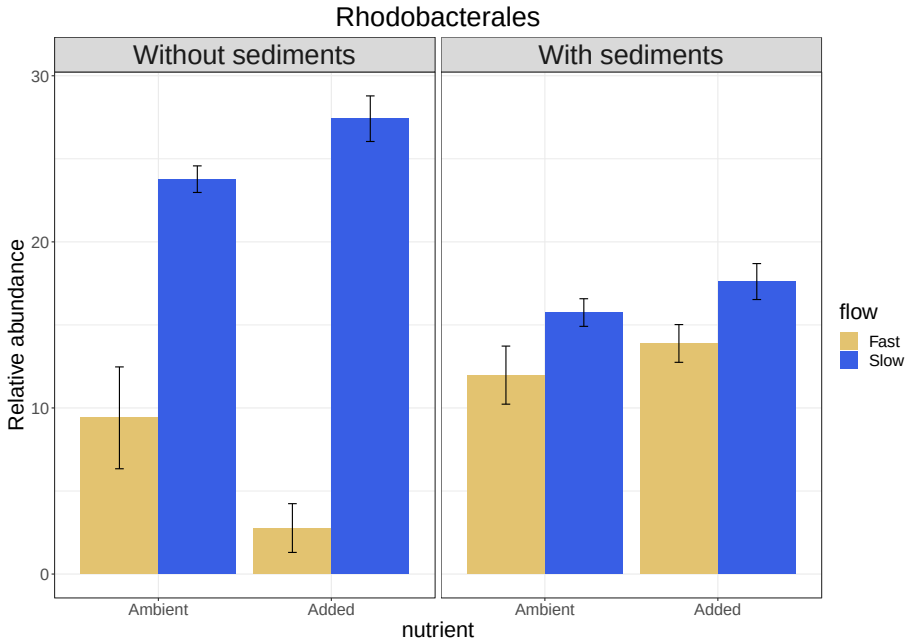


Figure S3.17: Rhodobacterales relative abundance interaction plot between the three stressors.

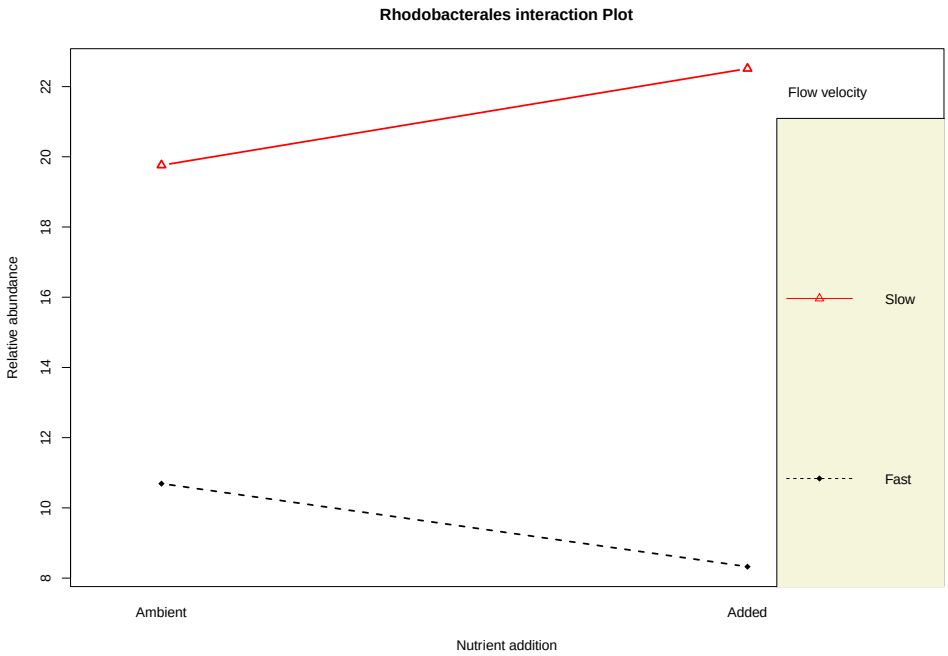


Figure S3.18: Rhodobacterales relative abundance interaction plot between the flow velocity reduction and nutrient treatment.

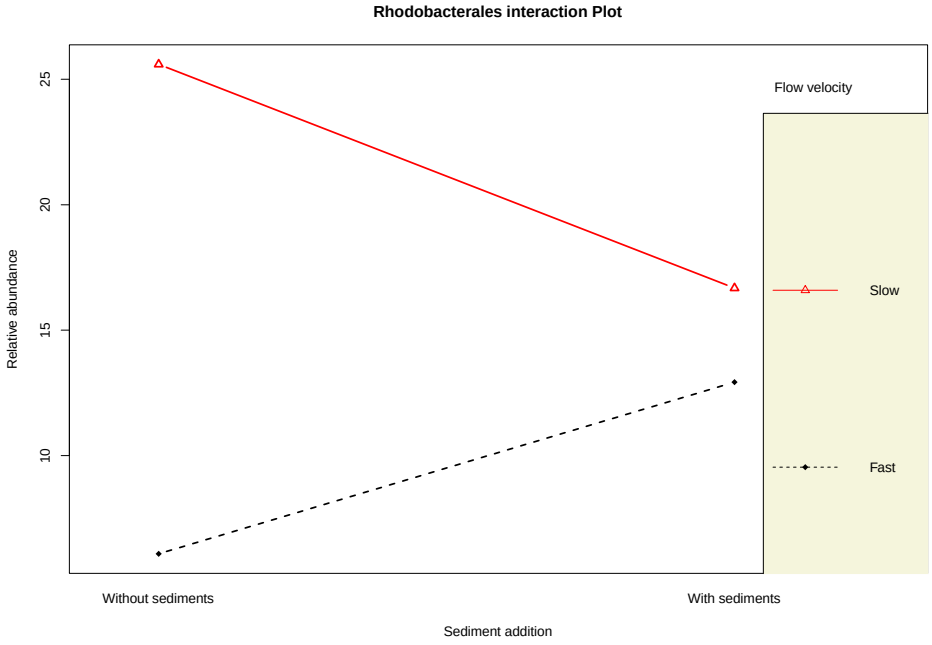


Figure S3.19: Rhodobacterales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

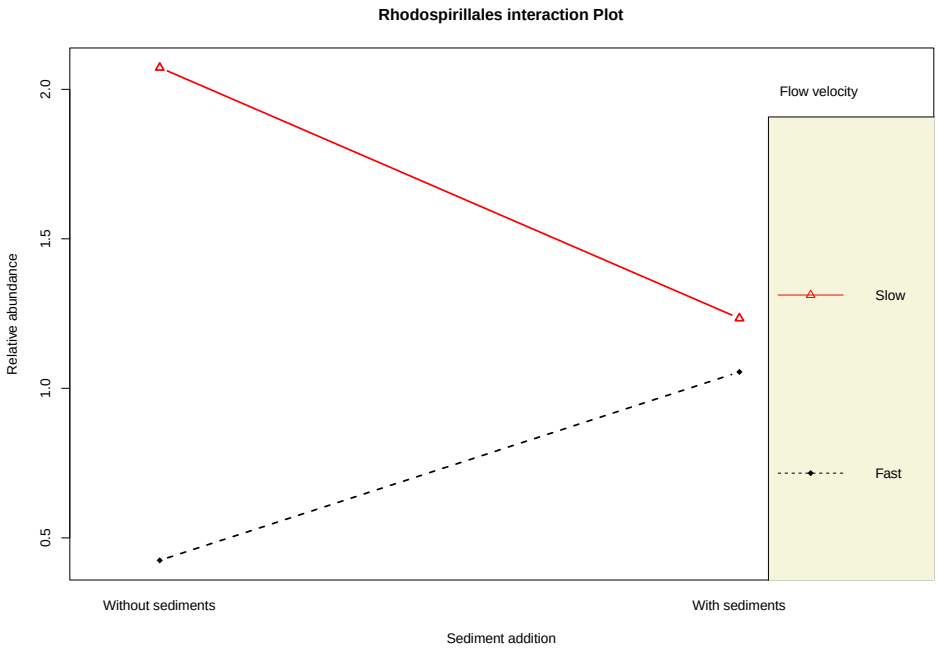


Figure S3.20: Rhodospirillales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

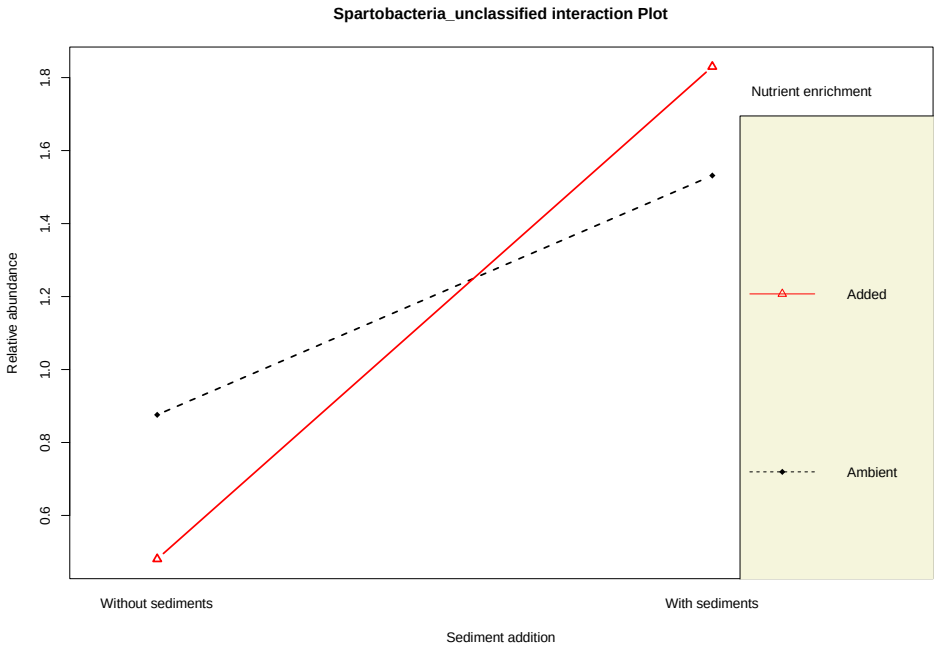


Figure S3.21: Spartobacteria_uncl relative abundance interaction plot between the sediment and nutrient treatment.

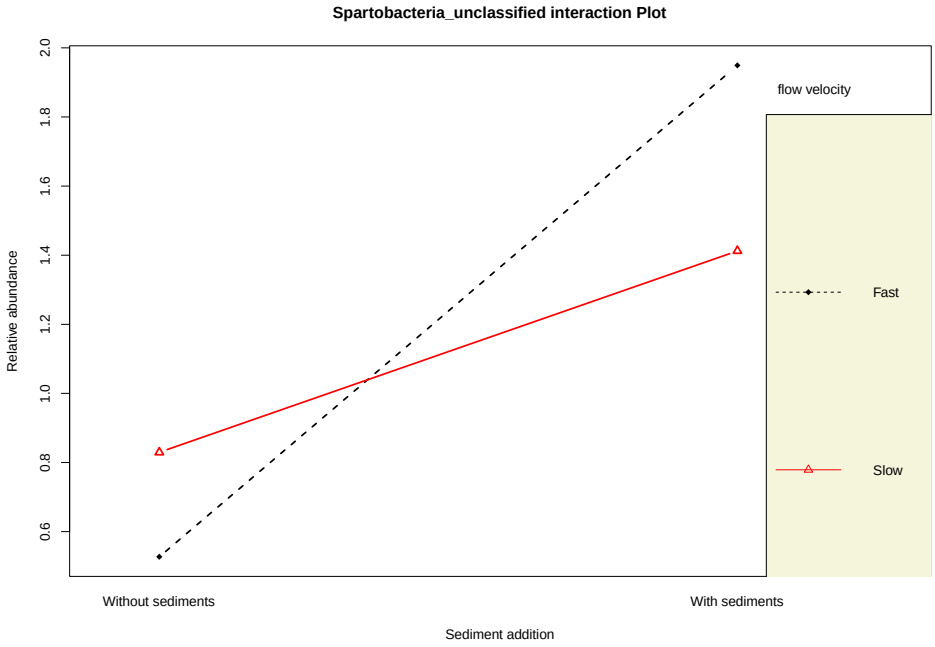


Figure S3.22: Spartobacteria_uncl relative abundance interaction plot between the flow velocity reduction and sediment treatment.

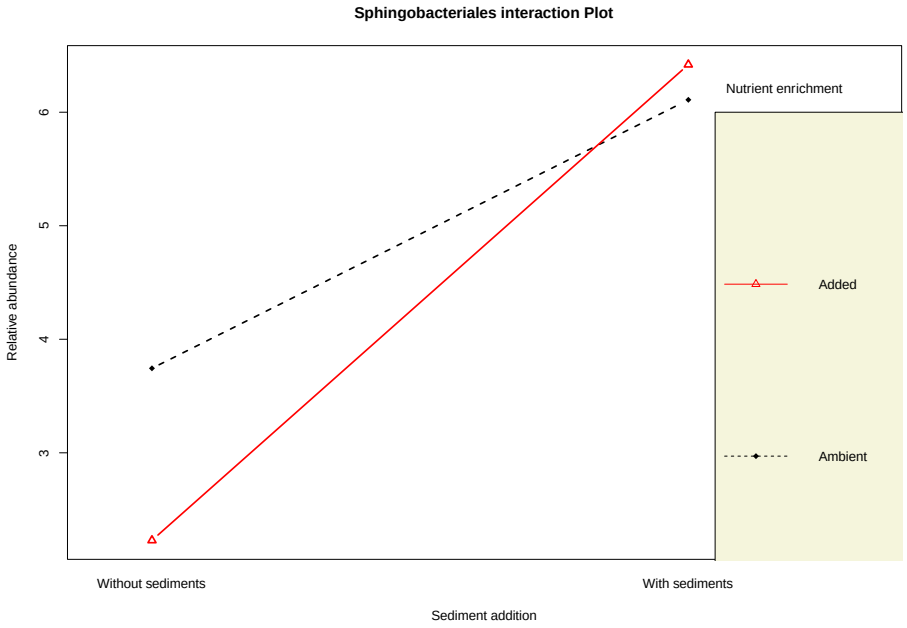


Figure S3.23: Sphingobacteriales relative abundance interaction plot between the sediment and nutrient treatment.

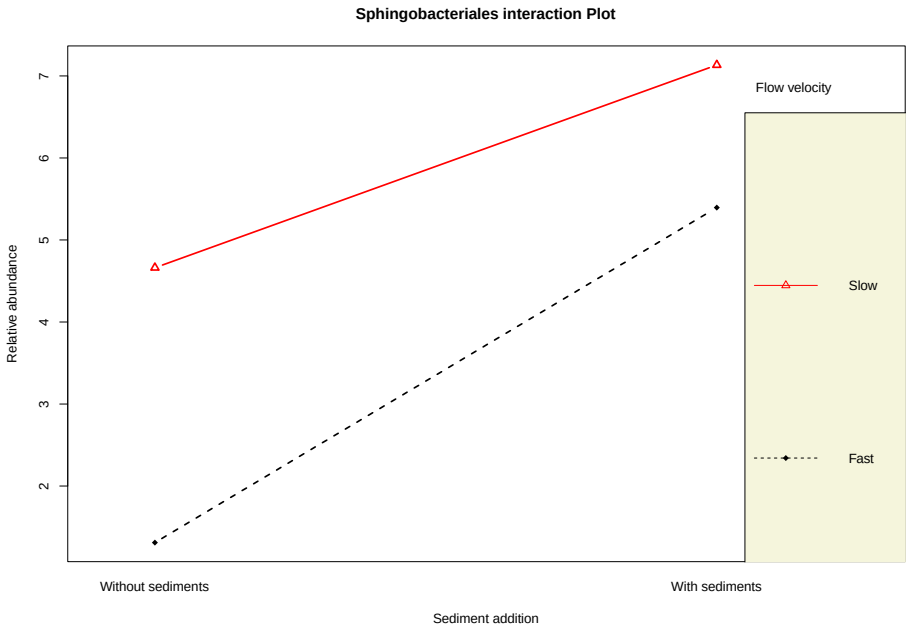


Figure S3.24: Sphingobacteriales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

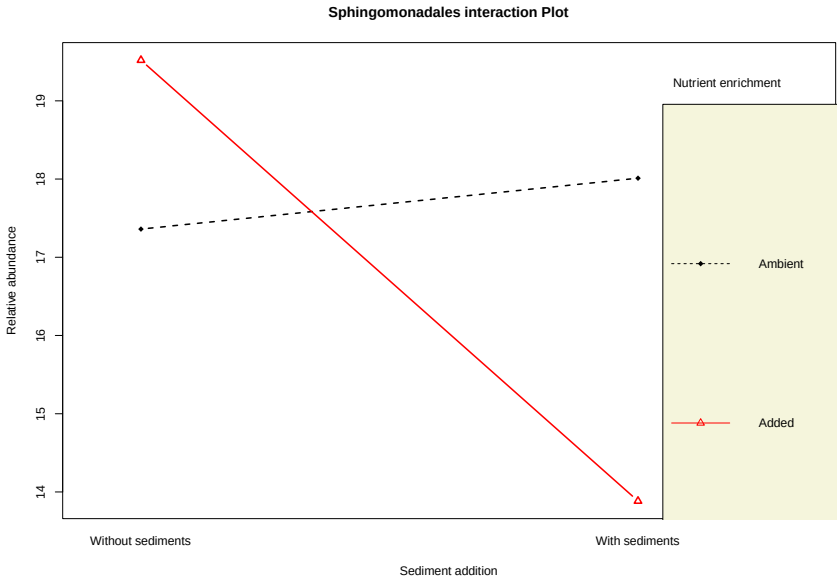


Figure S3.25: Sphingomonadales relative abundance interaction plot between the sedimentand nutrient treatment.

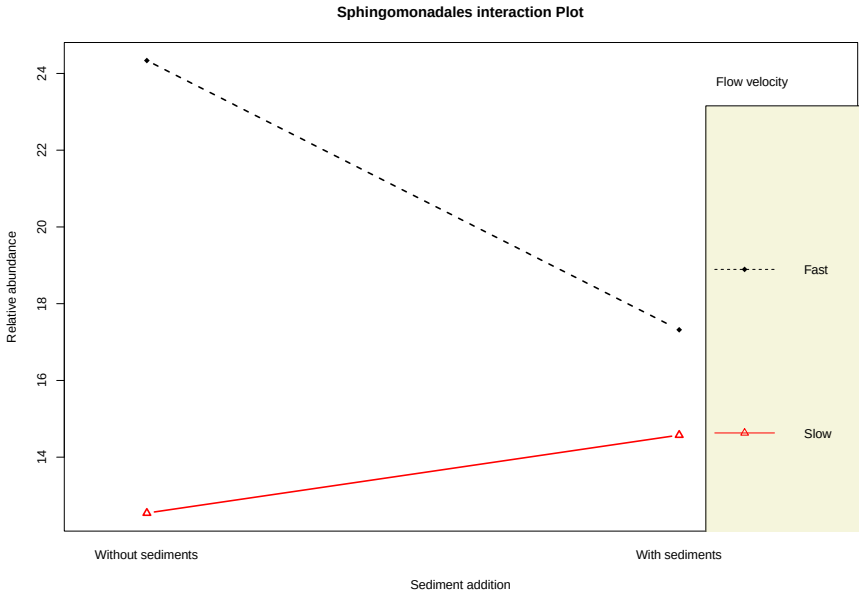


Figure S3.26: Sphingomonadales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

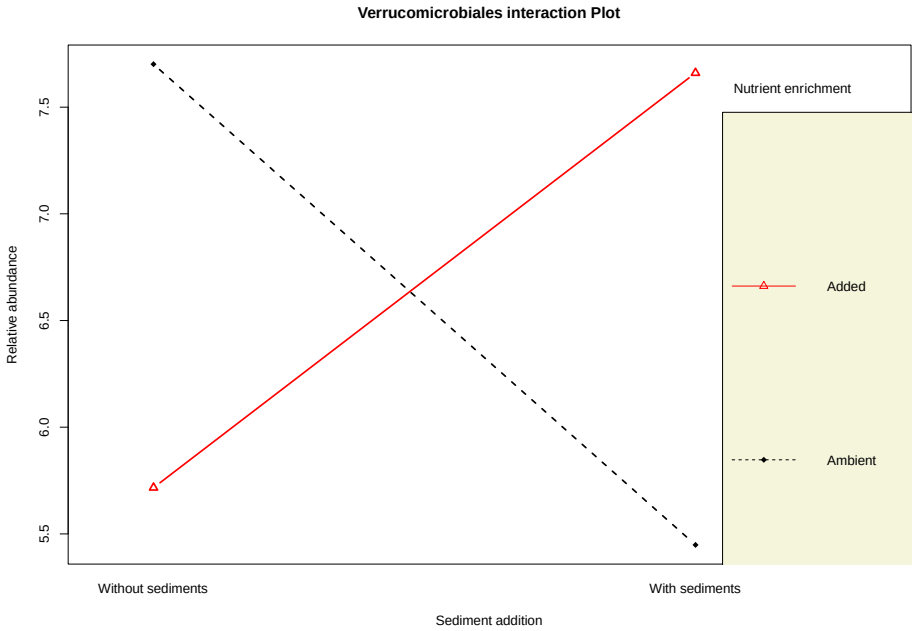


Figure S3.27: Verrucomicrobiales relative abundance interaction plot between the sediment and nutrient treatment.

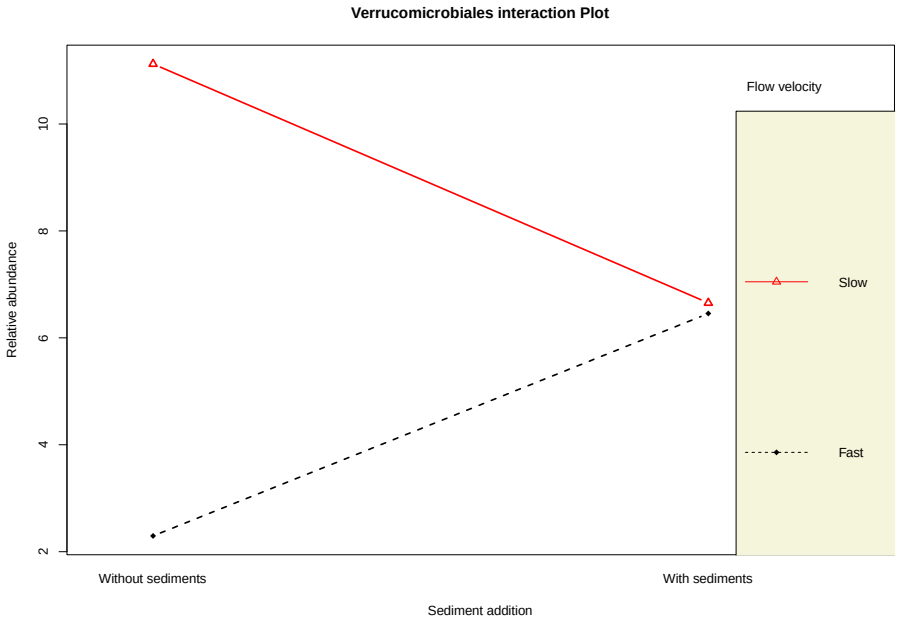


Figure S3.28: Verrucomicrobiales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

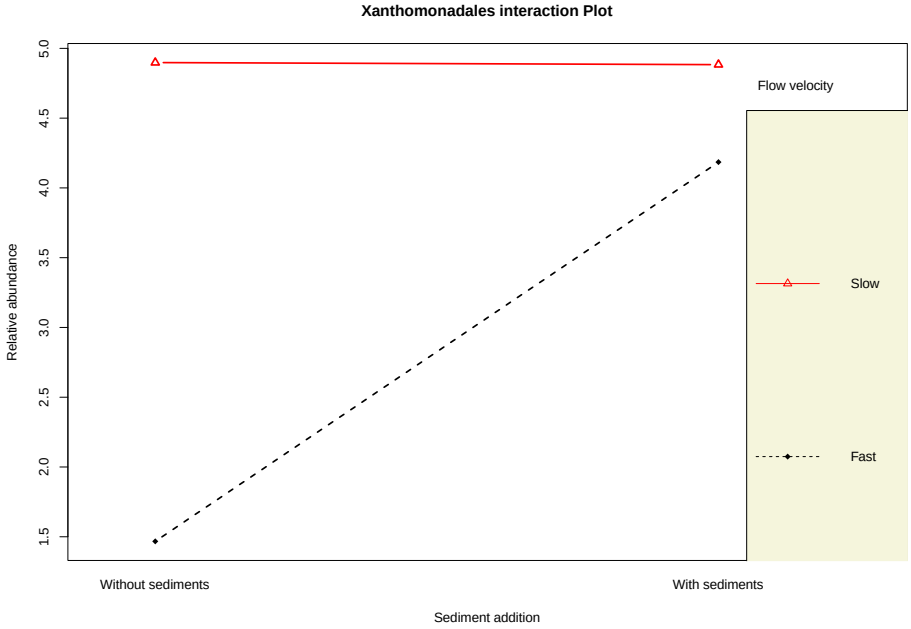


Figure S3.29: Xanthomonadales relative abundance interaction plot between the flow velocity reduction and sediment treatment.

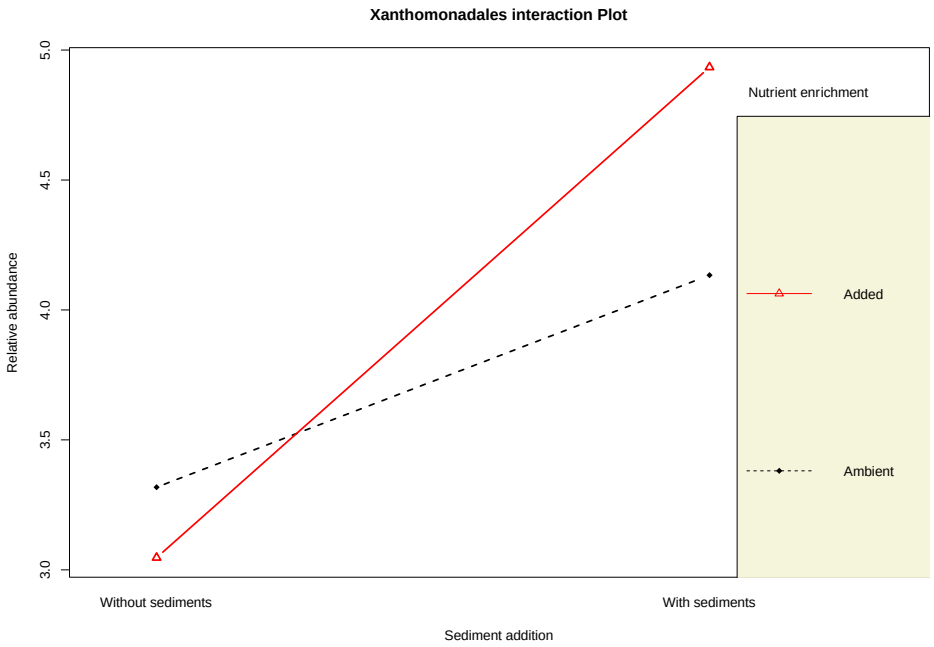


Figure S3.30: Xanthomonadales relative abundance interaction plot between the sediment and nutrient treatment.

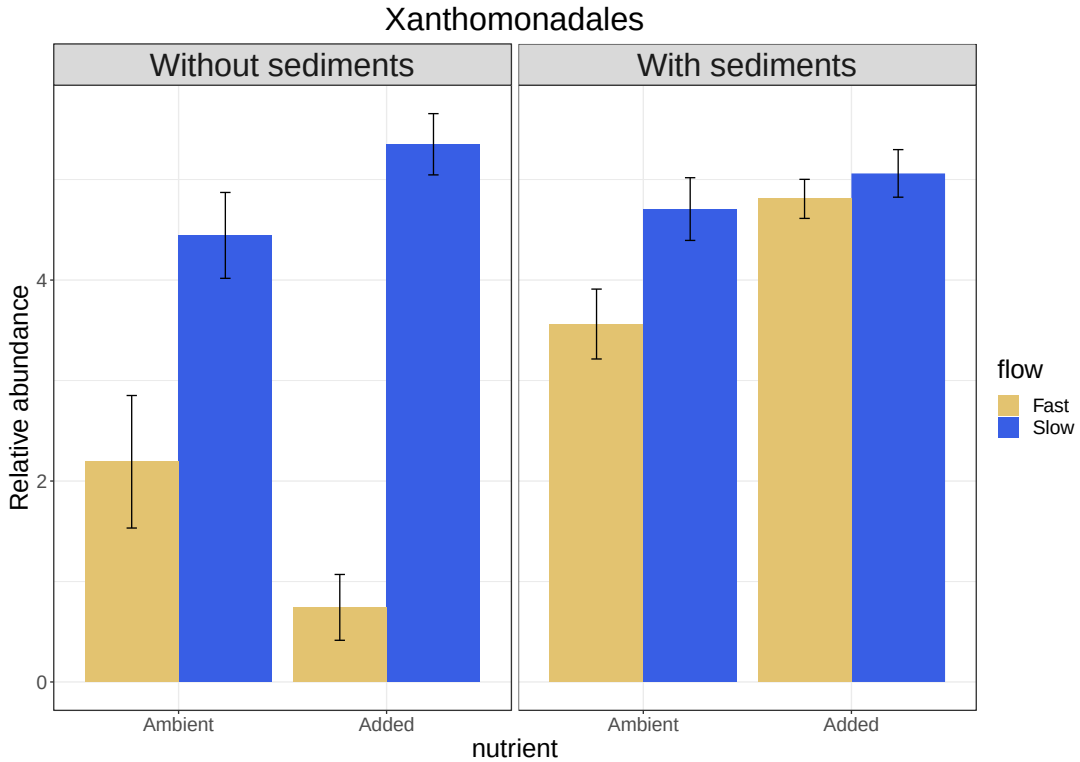


Figure S3.31: Xanthomonadales relative abundance interaction plot between the three stressors.

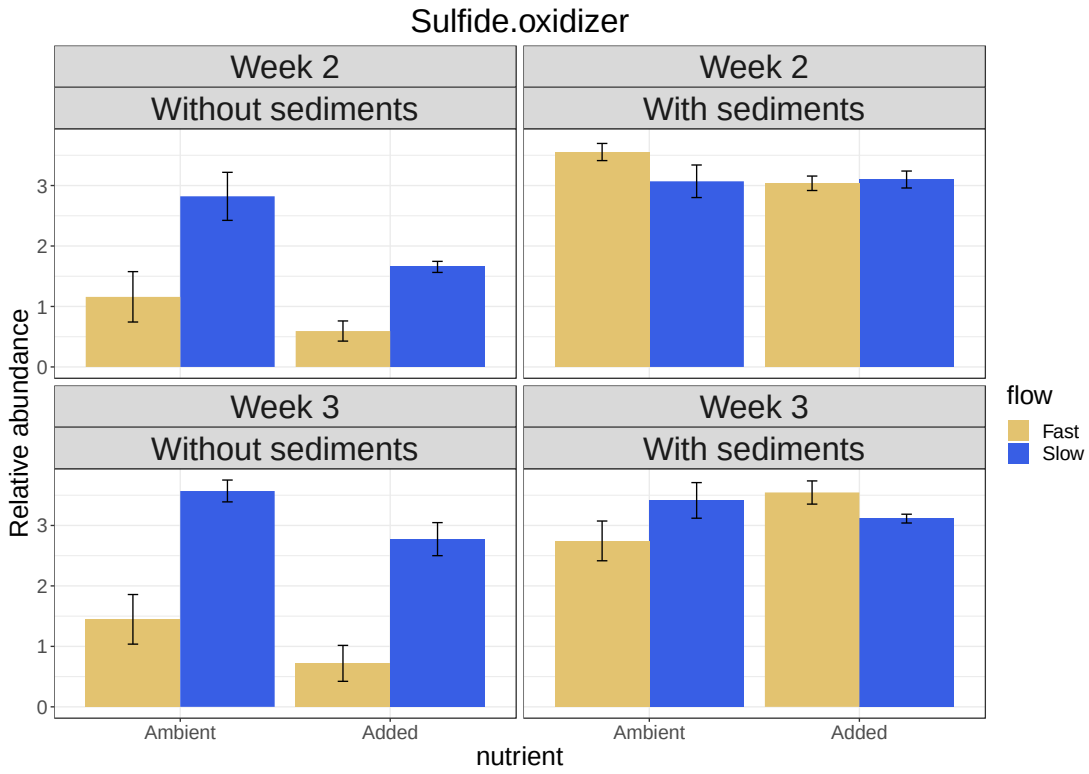


Figure S3.32: Sulfide oxidation predicted function relative abundance changes to the three different stressors over time.

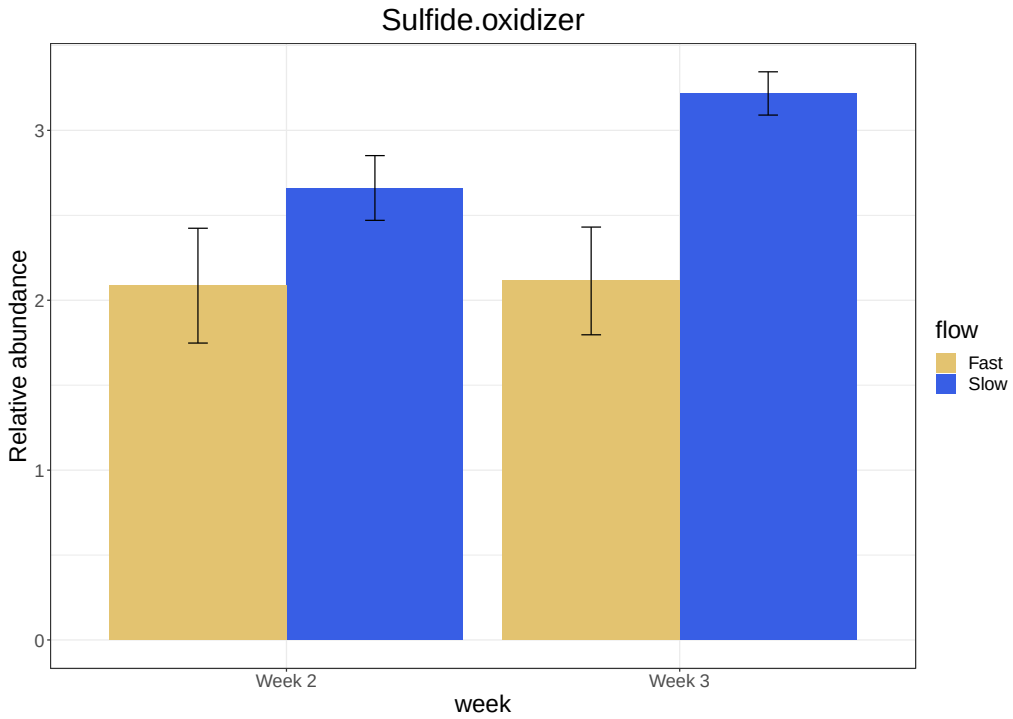


Figure S3.33: Sulfide oxidation predicted function relative abundance changes to the flow velocity reduction over time.

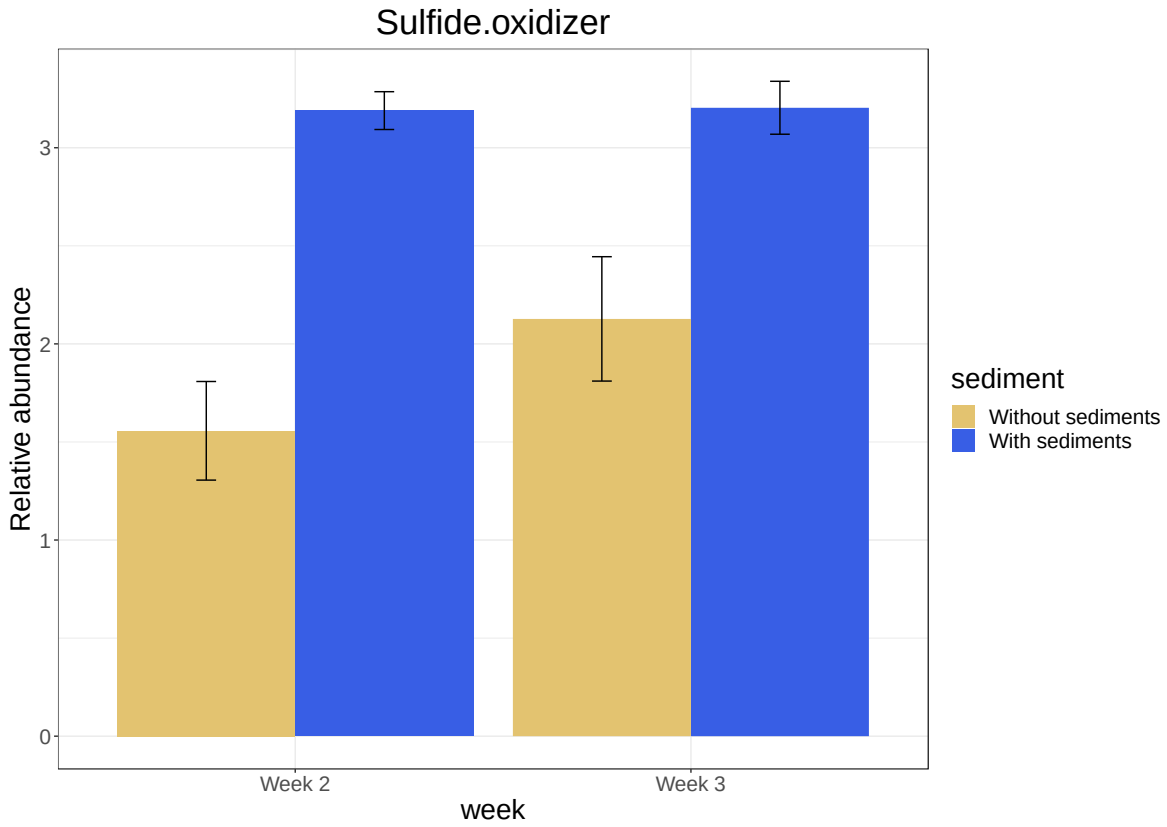


Figure S3.34: Sulfide oxidation predicted function relative abundance changes to the sediment addition over time.

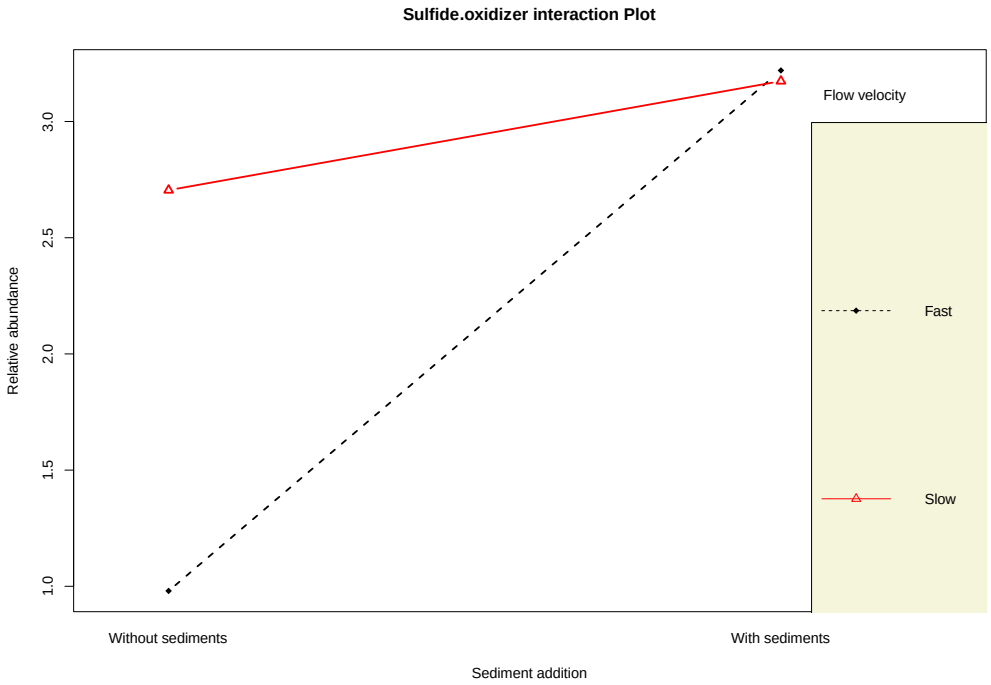


Figure S3.35: Sulfide oxidation predicted function relative abundance interaction plot between the sediment addition and flow velocity reduction stressors.

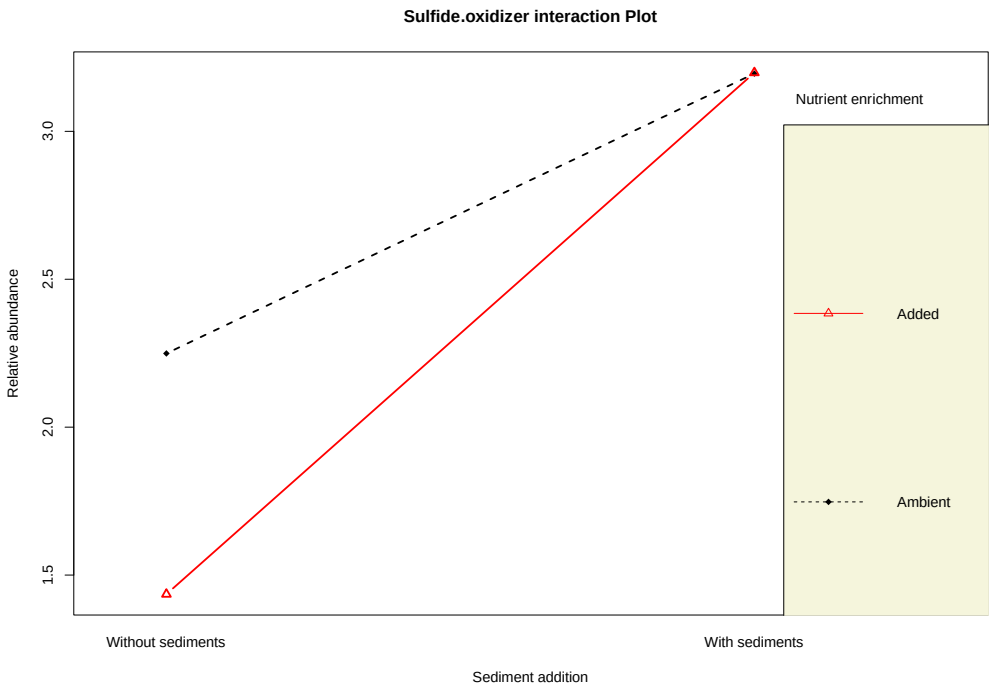


Figure S3.36: Sulfide oxidation predicted function relative abundance interaction plot between the sediment addition and the nutrient enrichment stressors.

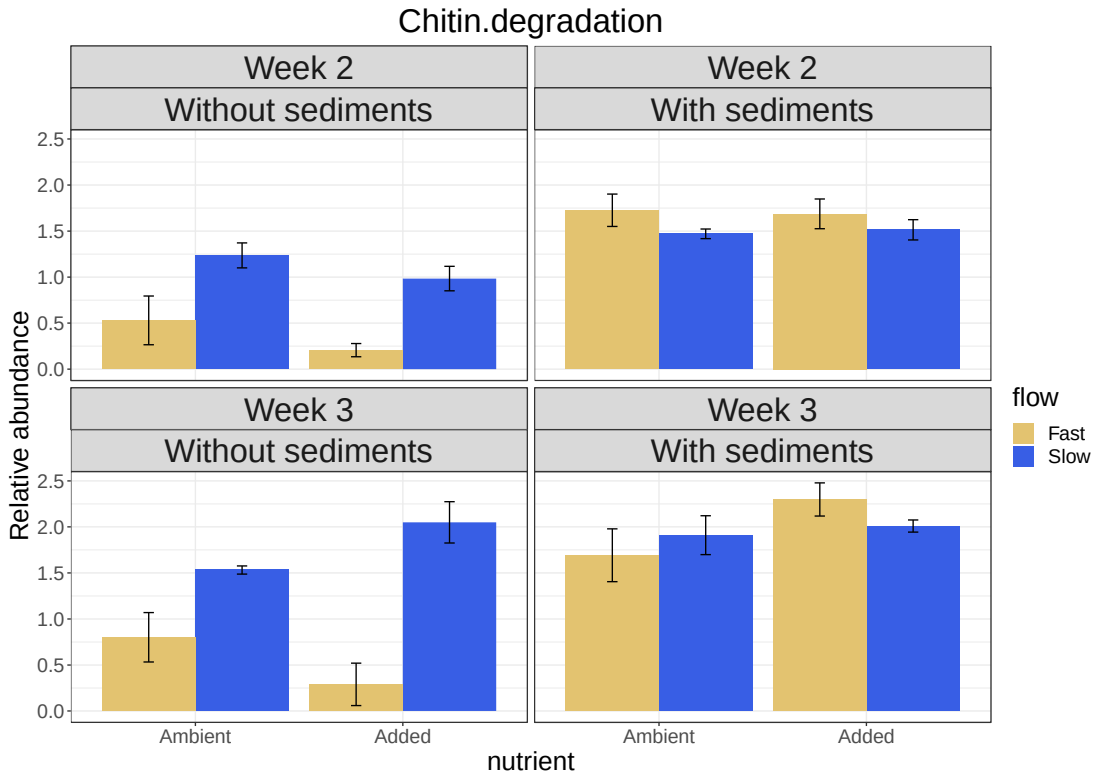


Figure S3.37: Chitin degradation predicted function relative abundance changes to the three different stressors over time.

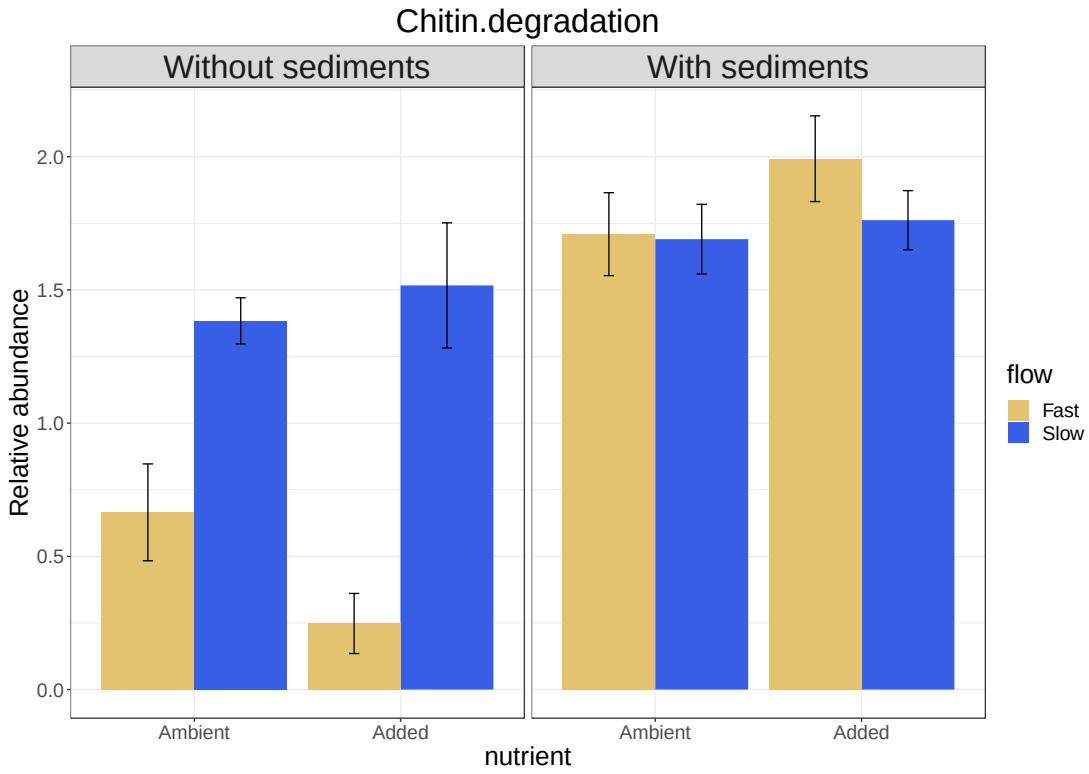


Figure S3.38: Chitin degradation predicted function relative abundance changes to the three stressors.

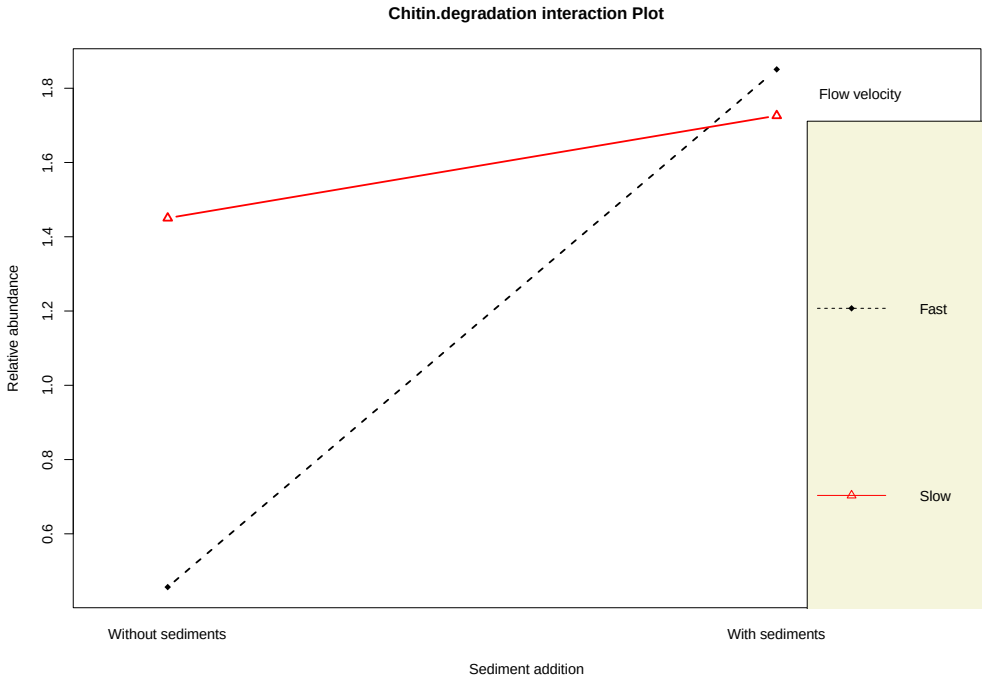


Figure S3.39: Chitin degradation predicted function relative abundance interaction plot between the sediment addition and the flow velocity reduction stressors.

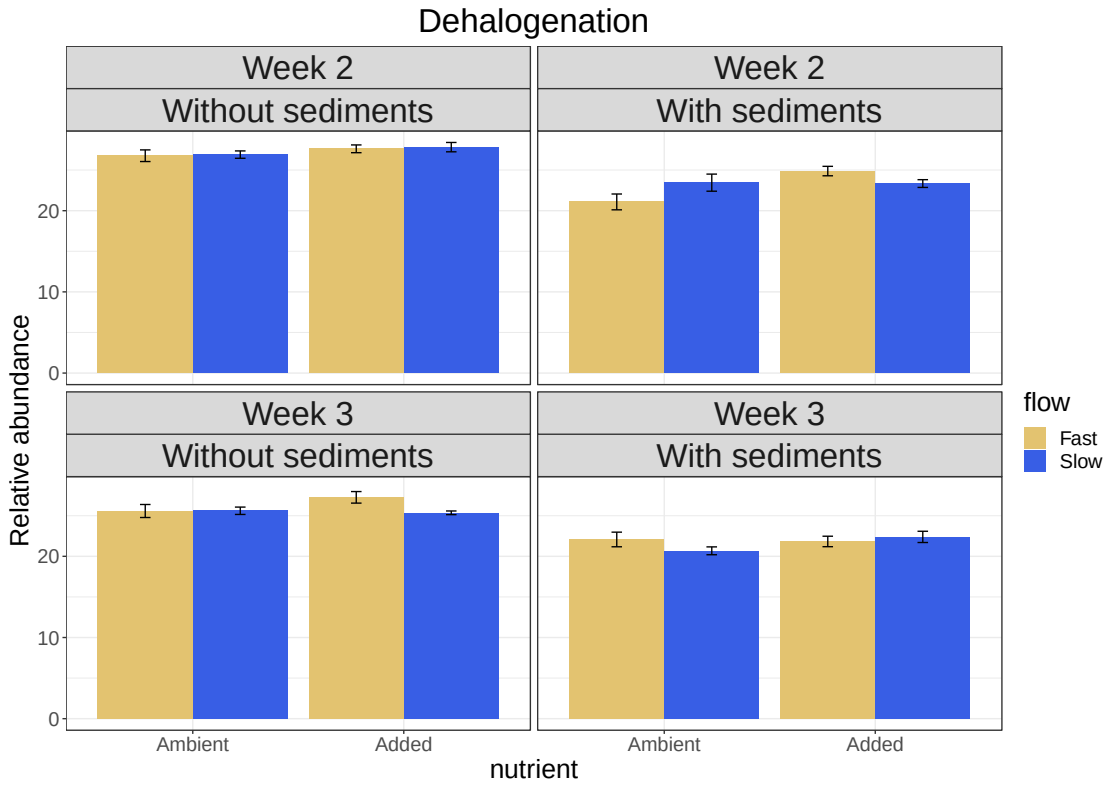


Figure S3.40: Dehalogenation predicted function relative abundance changes to the three different stressors over time.

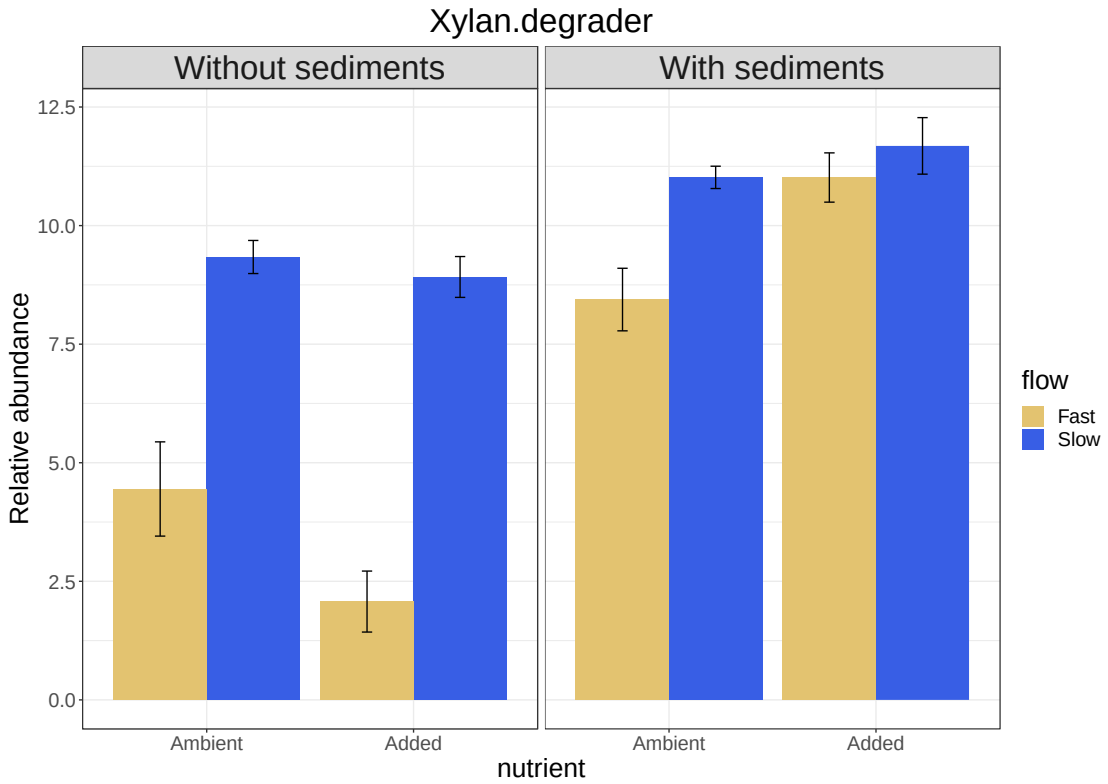


Figure S3.41: Xylan degradation predicted function relative abundance changes to the three different stressors.

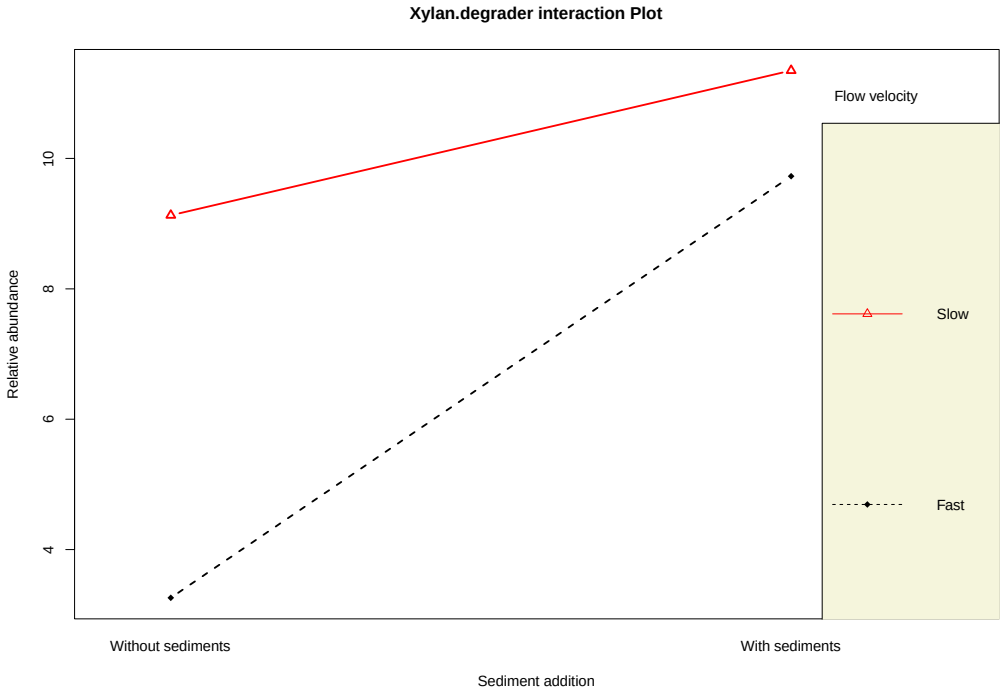


Figure S3.42: Xylan degradation predicted function relative abundance interaction plot between the sediment addition and the flow velocity reduction stressors.

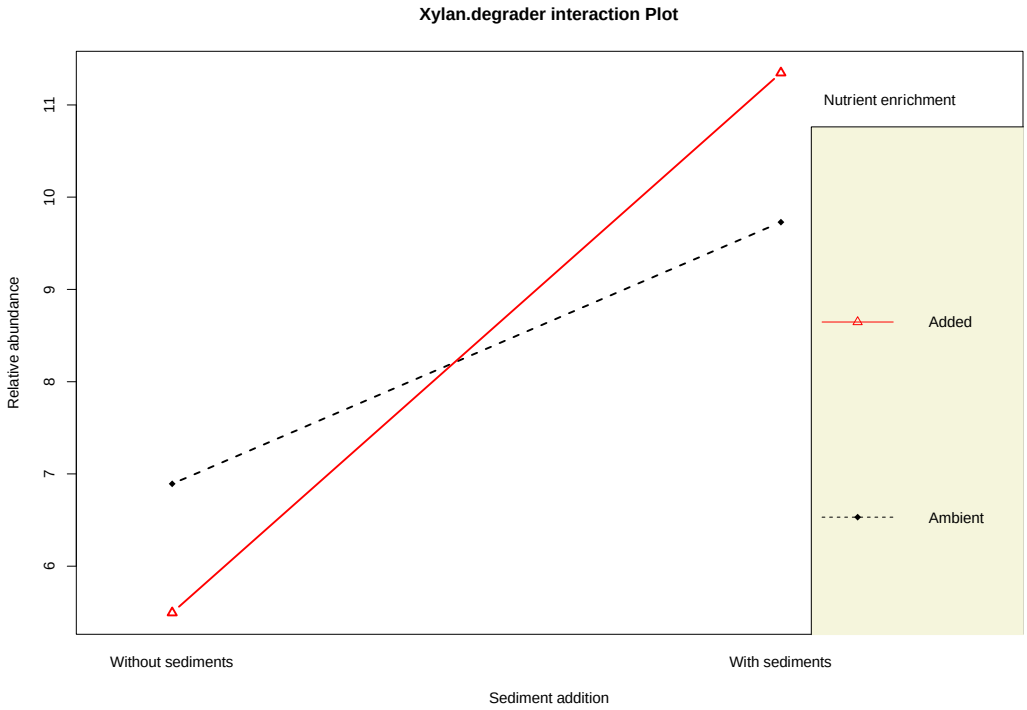


Figure S3.43: Xylan degradation predicted function relative abundance interaction plot between the sediment addition and nutrient enrichment stressors.

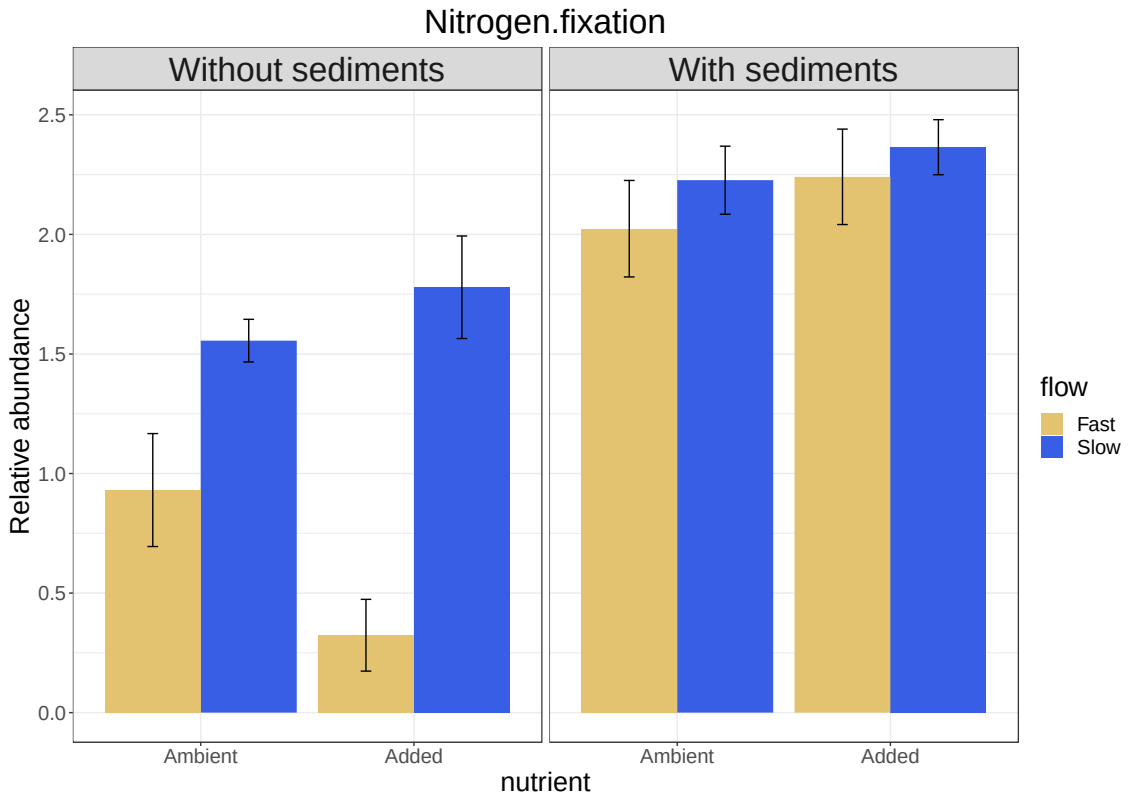


Figure S3.44: Nitrogen fixation predicted function relative abundance changes to the three different stressors.

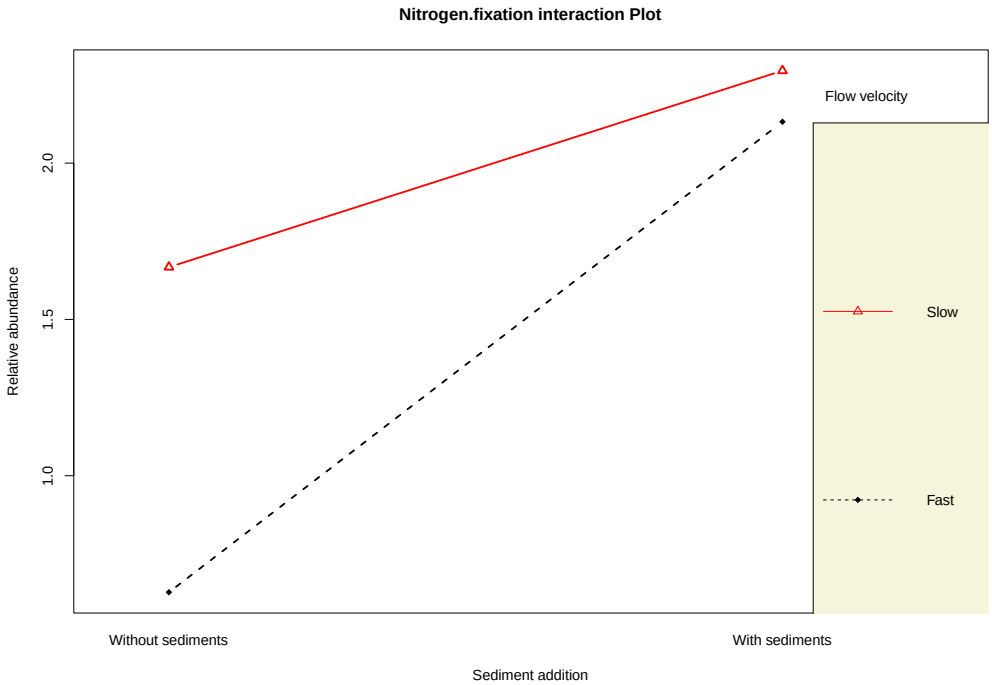


Figure S3.45: Nitrogen fixation predicted function relative abundance interaction plot between the sediment addition and the flow velocity reduction stressors.

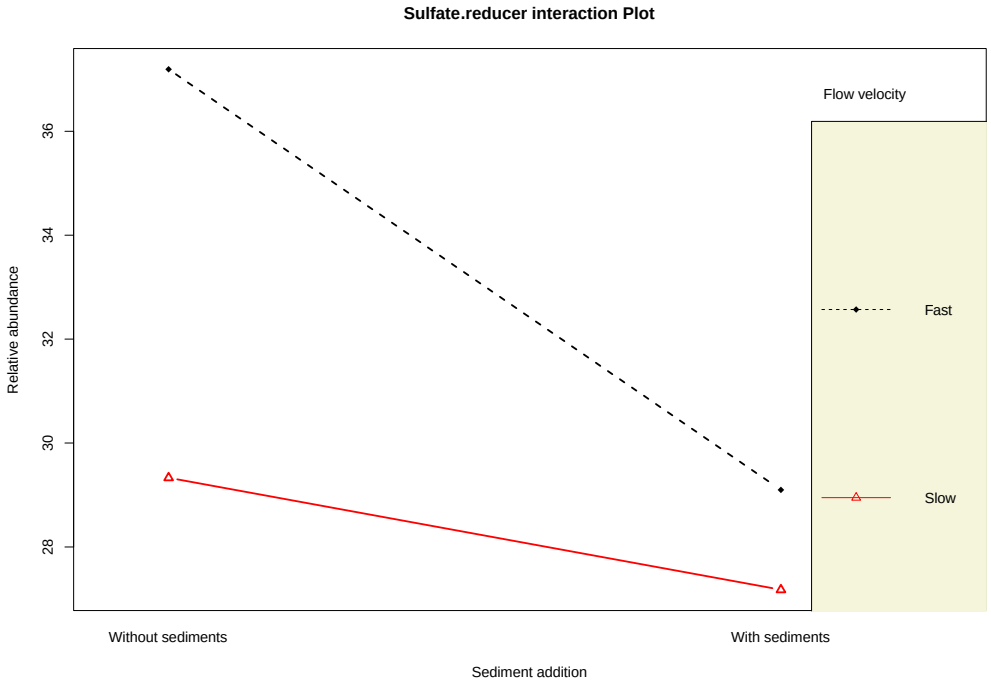


Figure S3.46: Sulfate reduction predicted function relative abundance interaction plot between the sediment addition and the flow velocity reduction stressors.

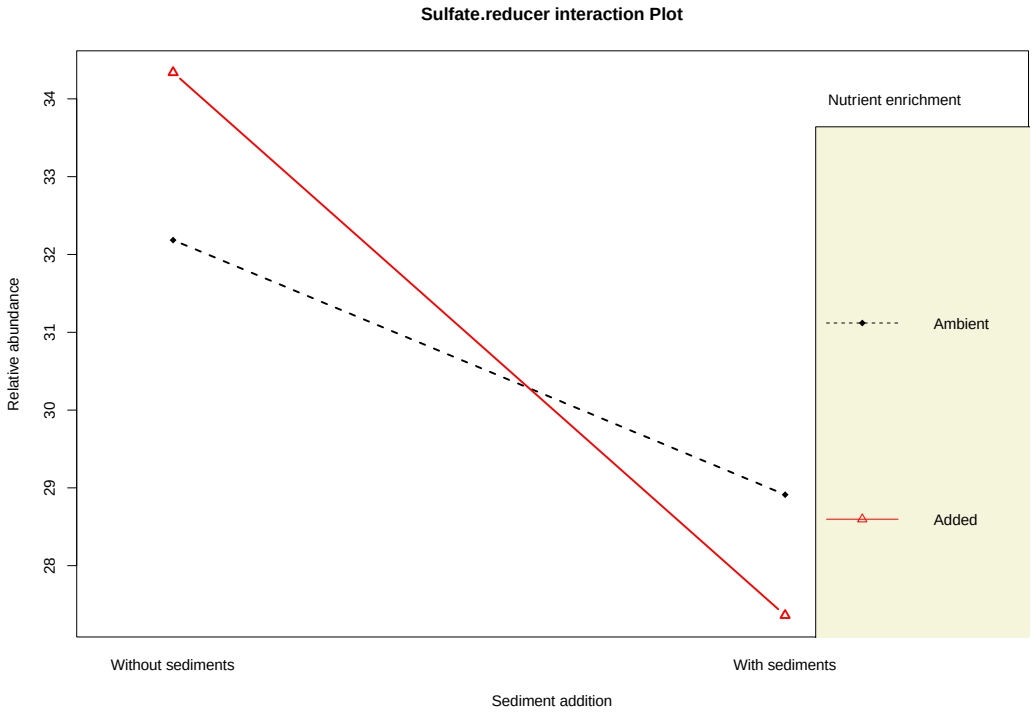


Figure S3.47: Sulfate reduction predicted function relative abundance interaction plot between the sediment addition and nutrient enrichment stressors.

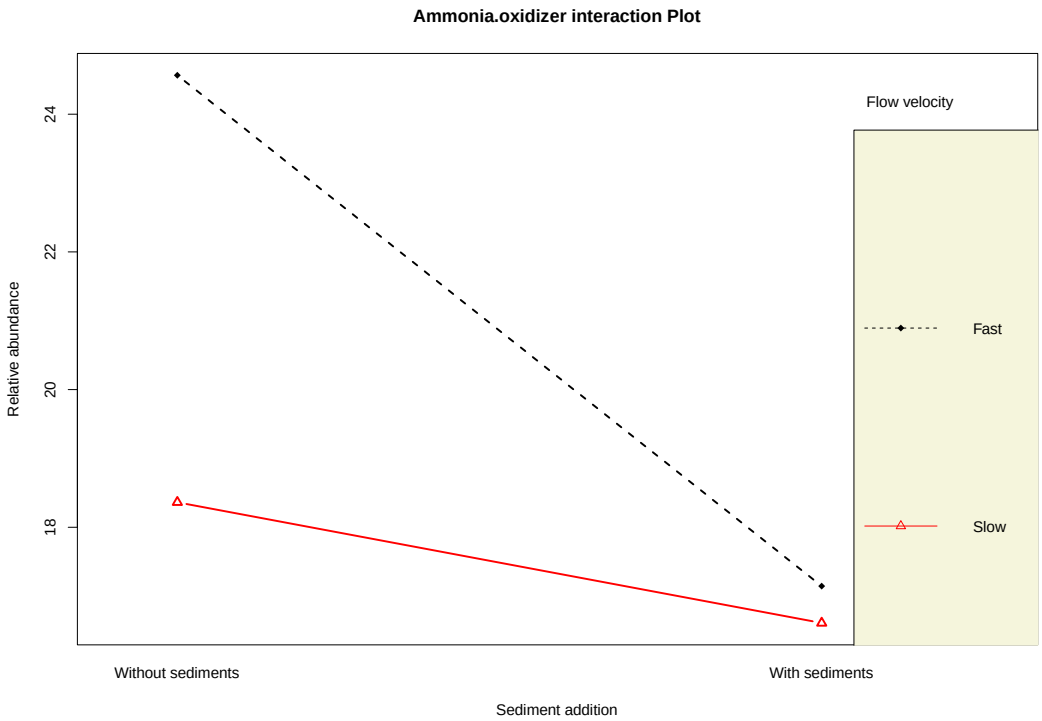


Figure S3.48: Ammonia oxidation predicted function relative abundance interaction plot between the sediment addition and the flow velocity reduction stressors.

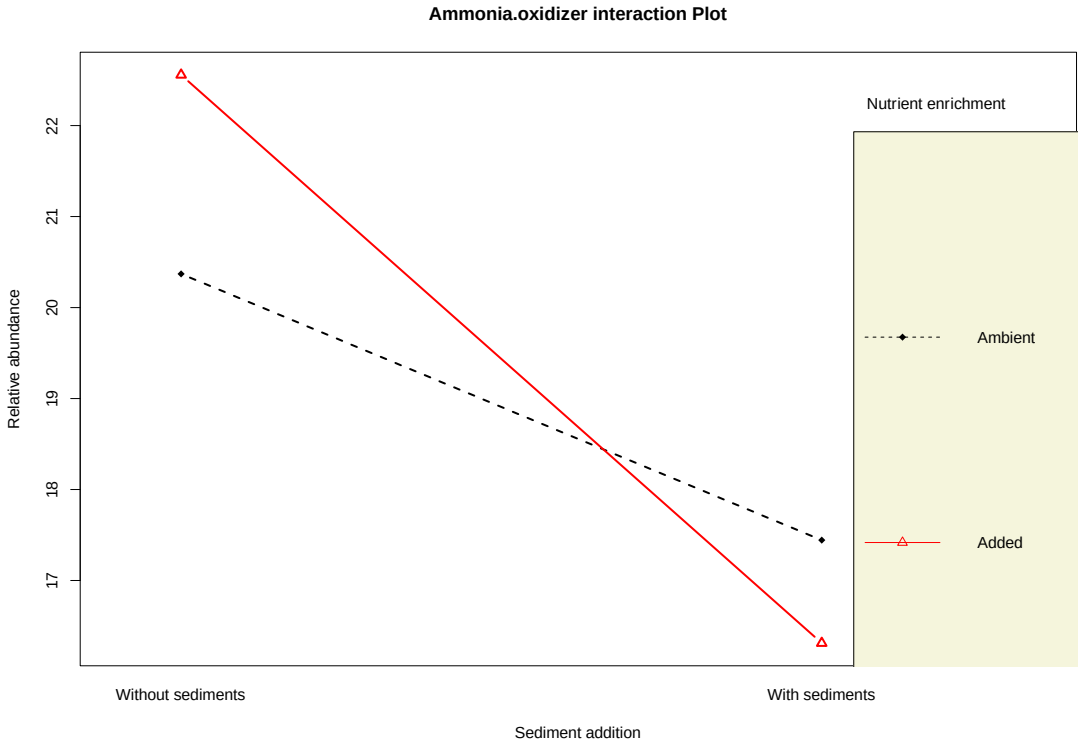


Figure S3.49: Ammonia oxidation predicted function relative abundance interaction plot between the sediment addition and nutrient enrichment stressors.

Table S3.1: SIMPER analysis results. The cumulative taxa accounted for >70% of the variation between the control treatment and the stressor treatments.

Week	Treatment	Taxon	Individual contribution	Cumulative contribution
2	Nutrient enrichment	*Pseudomonadales*	0.37	0.37
		Rhodobacterales	0.14	0.51
		Sphingomonadales	0.12	0.63
		Verrucomicrobiales	0.06	0.69
		Rhizobiales	0.05	0.74
	Sediment addition	*Pseudomonadales*	0.34	0.34
		Rhodobacterales	0.11	0.45
		Verrucomicrobiales	0.15	0.60
		Rhizobiales	0.04	0.64
		Planctomycetales	0.05	0.69
		Sphingobacteriales	0.04	0.73
	Flow reduction	*Pseudomonadales*	0.34	0.34
		Rhodobacterales	0.15	0.49
		Sphingomonadales	0.11	0.60
		Verrucomicrobiales	0.09	0.69
		Planctomycetales	0.05	0.74
	Nutrient + Sediment	*Pseudomonadales*	0.32	0.32
		Rhodobacterales	0.1	0.42
		Sphingomonadales	0.11	0.53
		Planctomycetales	0.09	0.62
		Verrucomicrobiales	0.08	0.70
		Rhizobiales	0.03	0.73
	Nutrient + Flow	*Pseudomonadales*	0.33	0.33
		Rhodobacterales	0.19	0.52
		Sphingomonadales	0.09	0.61
		Verrucomicrobiales	0.1	0.71
	Sediment + Flow	*Pseudomonadales*	0.33	0.33
		Rhodobacterales	0.12	0.45
		Sphingomonadales	0.1	0.55
		Verrucomicrobiales	0.06	0.61
		Planctomycetales	0.06	0.67
		Sphingobacteriales	0.06	0.73
	Nutrient + Flow + Sediment	*Pseudomonadales*	0.33	0.33
		Rhodobacterales	0.12	0.45
		Sphingomonadales	0.1	0.55
		Planctomycetales	0.06	0.61
		Verrucomicrobiales	0.07	0.68
		Sphingobacteriales	0.05	0.73
3	Nutrient enrichment	*Pseudomonadales*	0.37	0.37
		Rhodobacterales	0.11	0.48

		Sphingomonadales	0.11	0.59
		Rhizobiales	0.08	0.67
		Planctomycetales	0.05	0.72
	Sediment addition	*Pseudomonadales*	0.36	0.36
		Sphingomonadales	0.11	0.47
		Rhodobacterales	0.09	0.56
		Planctomycetales	0.07	0.63
		Rhizobiales	0.06	0.69
		Verrucomicrobiales	0.04	0.73
	Flow reduction	*Pseudomonadales*	0.34	0.34
		Rhodobacterales	0.15	0.49
		Sphingomonadales	0.11	0.60
		Verrucomicrobiales	0.08	0.68
		Planctomycetales	0.07	0.75
	Nutrient + Sediment	*Pseudomonadales*	0.37	0.37
		Sphingomonadales	0.1	0.47
		Rhodobacterales	0.07	0.54
		Planctomycetales	0.07	0.61
		Sphingobacteriales	0.06	0.67
		Rhizobiales	0.04	0.71
	Nutrient + Flow	*Pseudomonadales*	0.35	0.35
		Rhodobacterales	0.19	0.54
		Sphingomonadales	0.1	0.64
		Rhizobiales	0.05	0.69
		Planctomycetales	0.06	0.75
	Sediment + Flow	*Pseudomonadales*	0.4	0.40
		Rhodobacterales	0.08	0.48
		Sphingomonadales	0.08	0.56
		Sphingobacteriales	0.07	0.63
		Rhizobiales	0.06	0.69
		Planctomycetales	0.06	0.75
	Nutrient + Flow + Sediment	*Pseudomonadales*	0.38	0.38
		Sphingomonadales	0.1	0.48
		Rhodobacterales	0.09	0.57
		Planctomycetales	0.09	0.66
		Sphingobacteriales	0.05	0.71

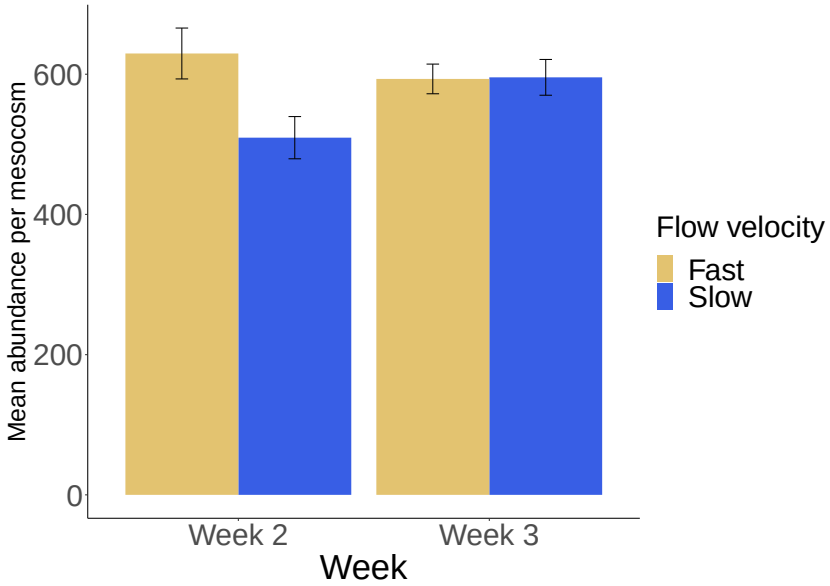


Figure S4.1: Average number of benthic invertebrates in the two flow velocity treatments on the two sampling occasions (Error Bars = +/- SE, *n* = 16).

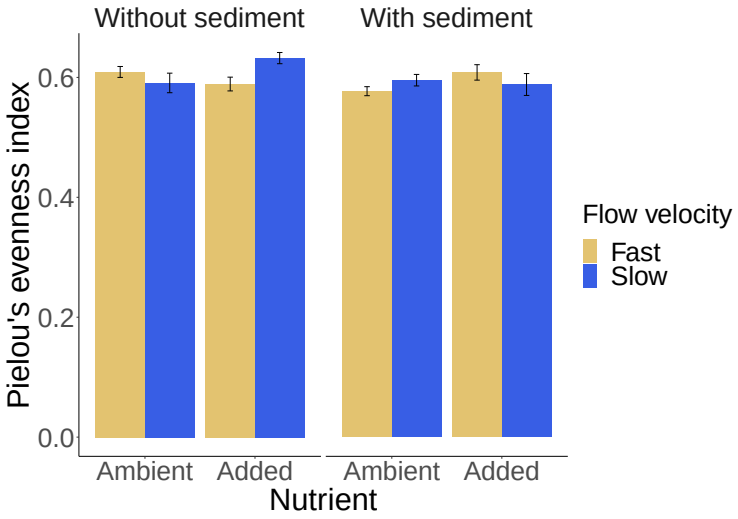


Figure S4.2: Average evenness (Pielou's) of the benthic invertebrate community across the experimental treatments. (Error Bars = +/- SE, *n* = 8 per treatment combination).

Common invertebrate taxa stressor interactions:

Numbers of Nemouridae (Fig. S4.3) and dipteran pupae (Fig. S4.4) remained stable or increased when sediment alone was added but decreased when flow velocity was reduced as well. For Nemouridae, this interaction overrode the weaker, negative sediment main effect (Table 4.3). Further, the negative effect of flow velocity reduction on Baetidae was stronger in the absence of fine sediment (Fig S4.5). Ephemerellidae increased in abundance in nutrient-enriched mesocosms at fast flow but decreased when enrichment was combined with reduced flow velocity (Fig S4.6). Finally, Chironomidae abundance increased with reduced flow velocity in non-enriched and sediment-free channels, whereas they decreased (or were unaffected) with reduced flow velocity in all other treatment combinations (Fig S4.7). By

contrast, Gordiidae abundance was highest when nutrients were added and flow velocity was reduced in the absence of sediment, but lowest when all three stressors were applied (Fig. S4.8).

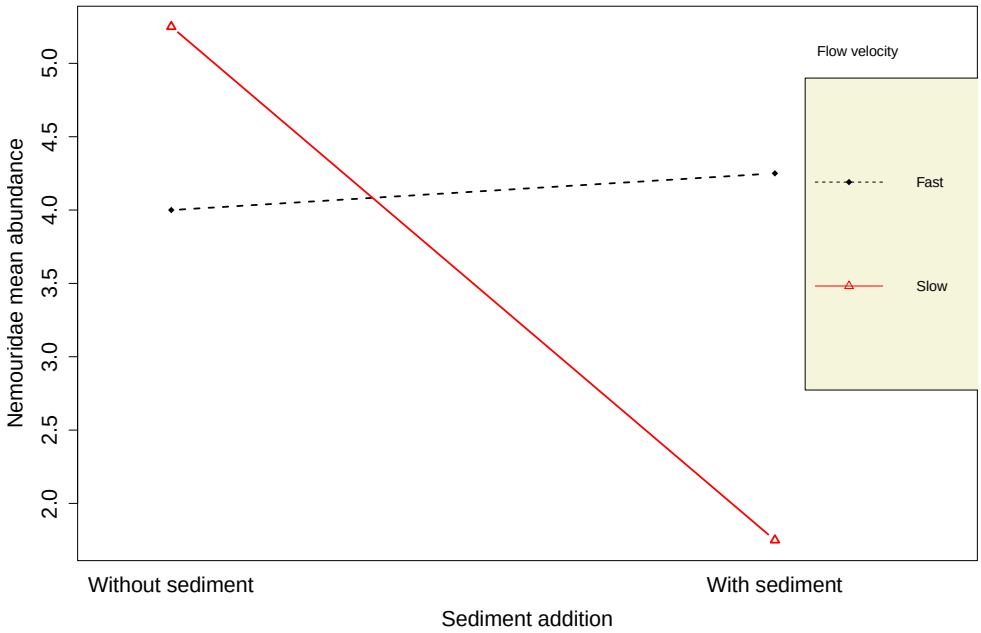


Figure S4.3: Interactive effects of flow velocity reduction and sediment addition on the abundance of Nemouridae.

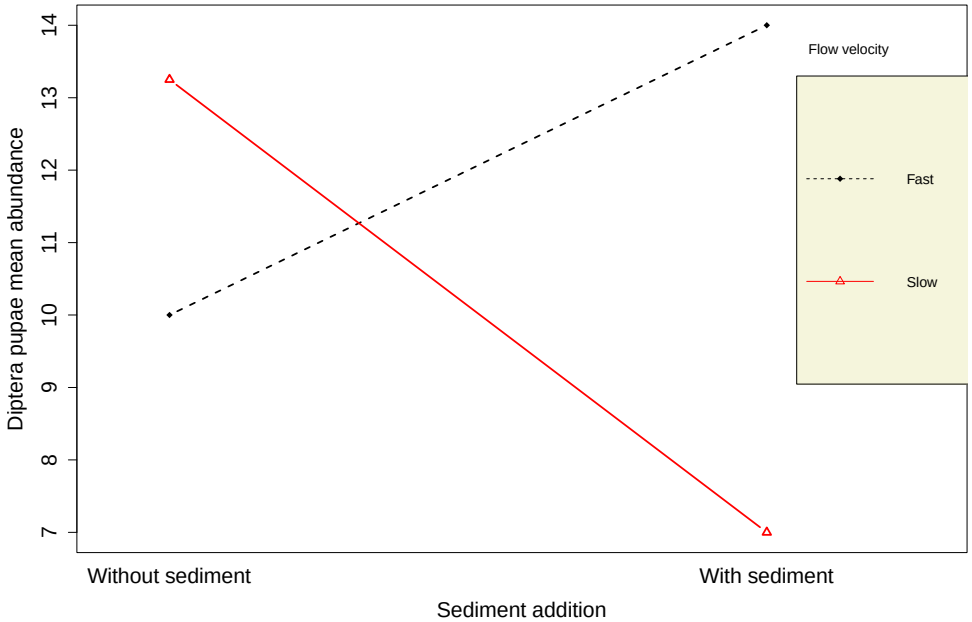


Figure S4.4: Interactive effects of flow velocity reduction and sediment addition on the abundance of Diptera pupae.

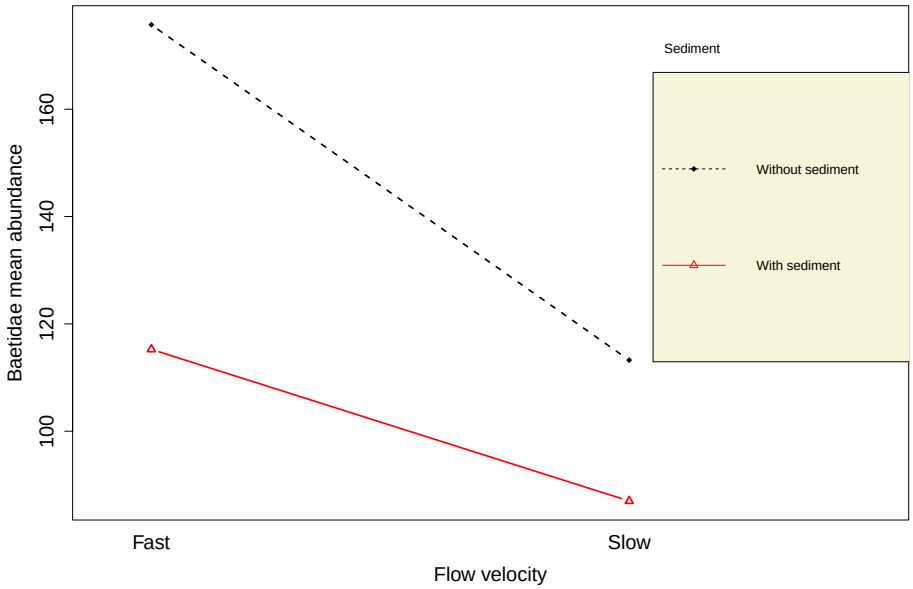


Figure S4.5: Interactive effects of flow velocity reduction and sediment addition on the abundance of Baetidae.

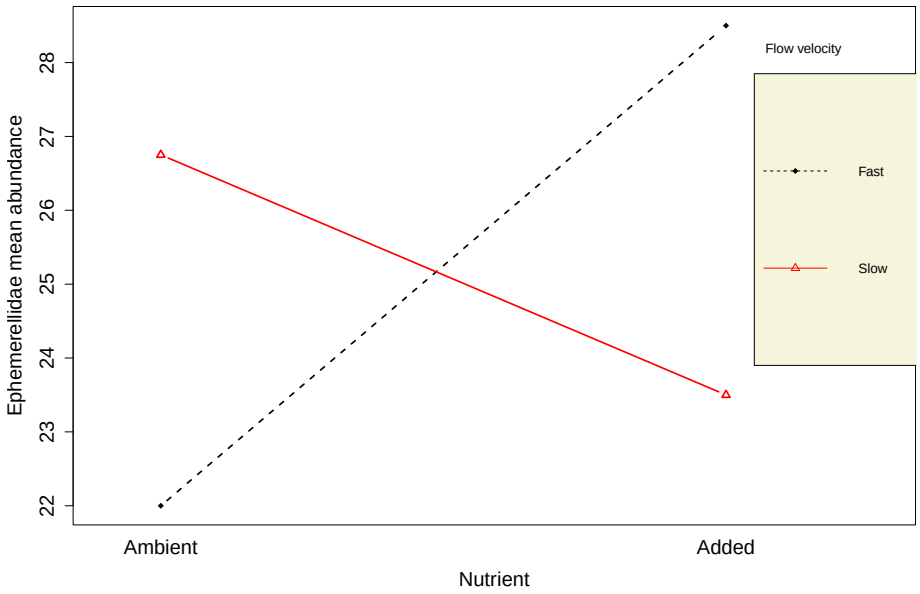


Figure S4.6: Interactive effects of flow velocity reduction and nutrient enrichment on the abundance of Ephemerillidae.

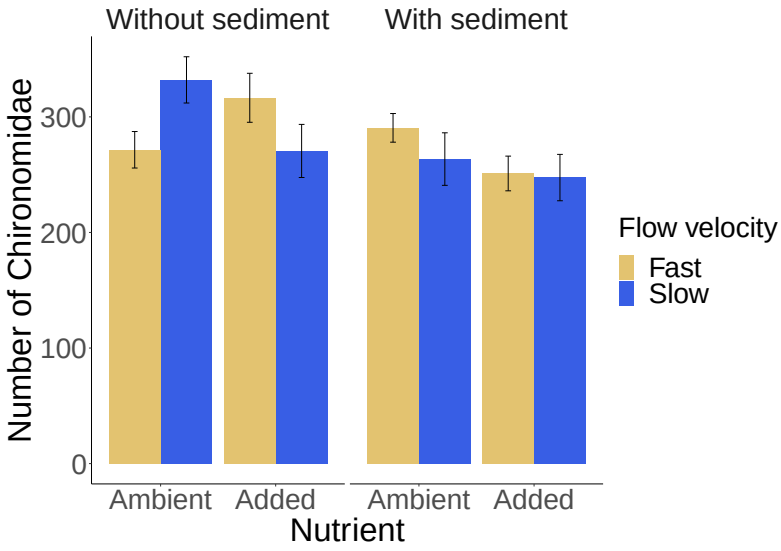


Figure S4.7: Average number of Chironomidae in the two flow velocity treatments on the two sampling occasions (Error Bars = +/- SE, *n* = 8).

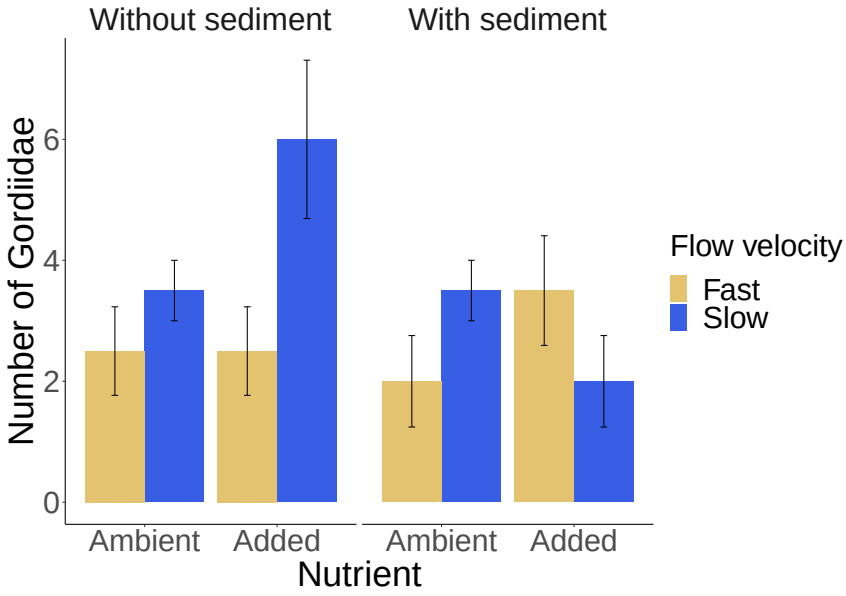


Figure S4.9: Average number of Gordiidae in the two flow velocity treatments on the two sampling occasions (Error Bars = +/- SE, *n* = 8).

Stressor effects on functional diversity and traits

Interactive stressor effects on trait variables were fairly rare, but interactions between sediment and current velocity reduction as well as between nutrients and velocity reduction were seen in the relative abundances of the two morphological traits and those of shredders, respectively. When flow velocity was reduced, the proportion of streamlined individual generally decreased; however, the slope was smaller in mesocosms with sediment added (Fig. S4.11). The opposite pattern was seen for non-streamlined organisms (Fig. S4.12). For shredders, relative abundance

decreased when nutrients were added at fast flow velocities but this pattern was reversed at reduced flow velocity (Fig. S4.13).

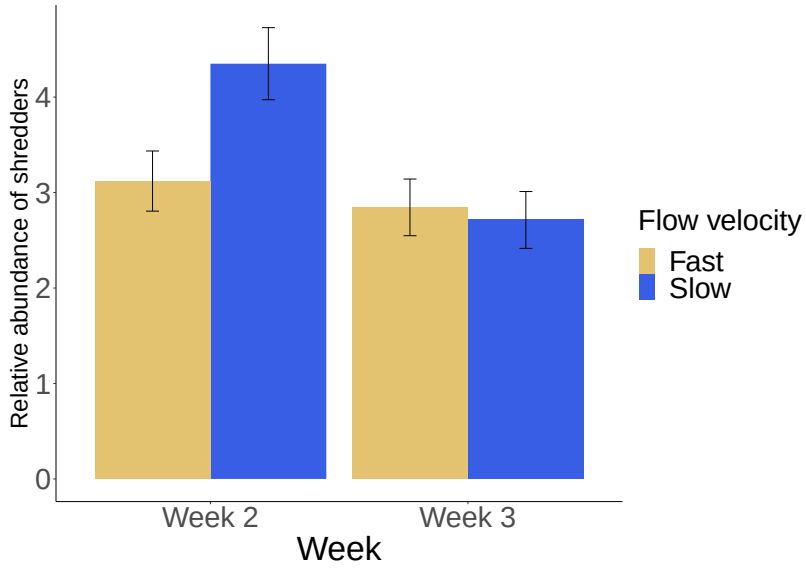


Figure S4.10: Flow velocity main effects across sampling dates on the average relative abundance of shredding invertebrates (Error Bars = +/- SE, n=16).

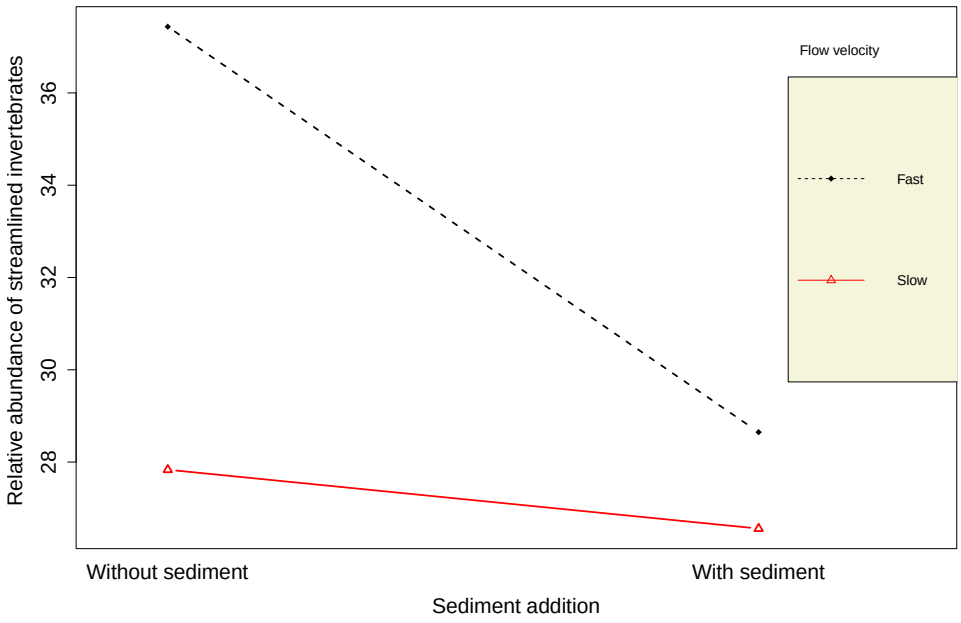


Figure S4.11: Interactive effects of flow velocity reduction and sediment addition on the relative abundance of streamlined macroinvertebrates.

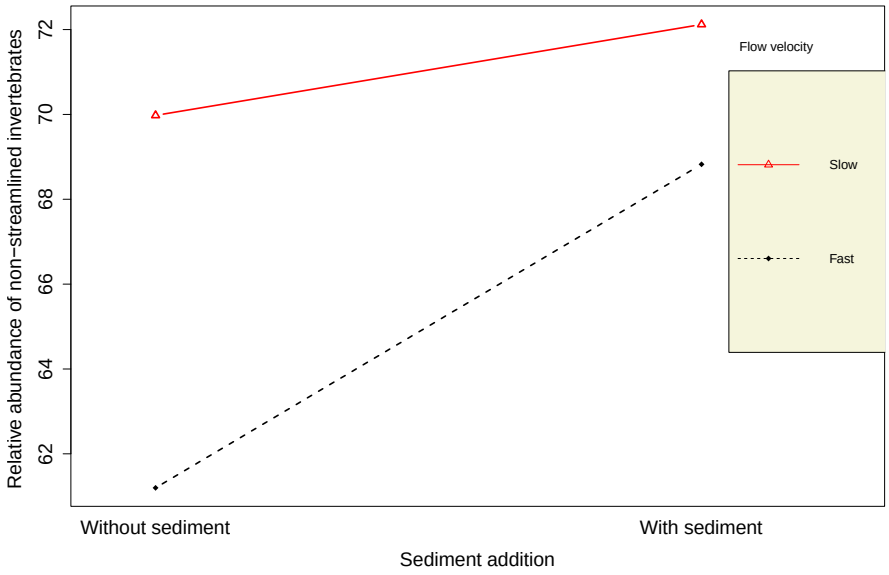


Figure S4.12: Interactive effects of flow velocity reduction and sediment addition on the relative abundance of non-streamlined macroinvertebrates.

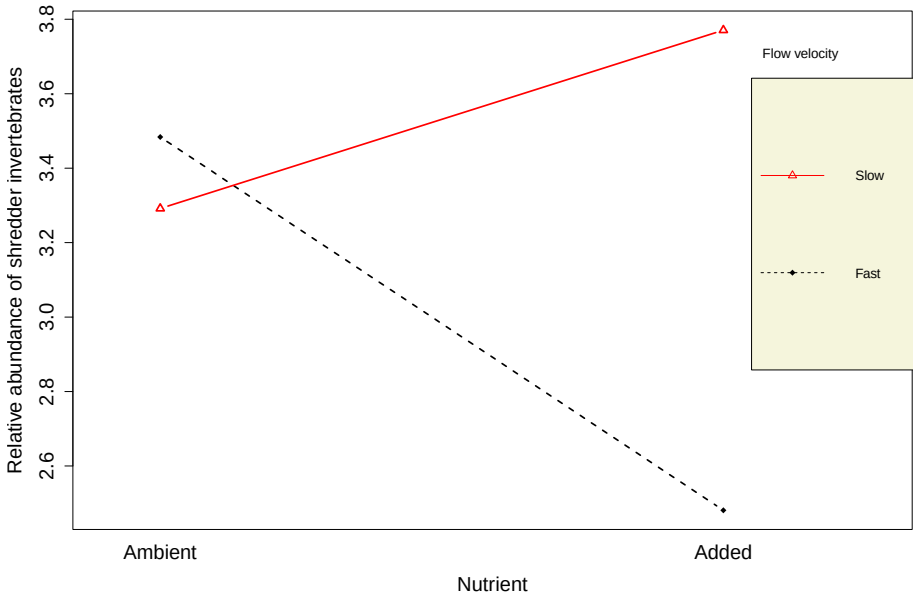


Figure S4.13: Interactive effects of flow velocity reduction and nutrient enrichment on the relative abundance of shredding macroinvertebrates.