CUMULATIVE EFFECTS OF MULTIPLE AGRICULTURAL STRESSORS ON FRESHWATER ECOSYSTEMS

by

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Abstract

Agriculture is the primary cause of sedimentation, nutrient enrichment, and insecticide contamination of freshwater ecosystems. Despite the widespread co-occurrence of these ecological stressors, little is known about their potential interactive effects. I conducted three experiments manipulating combinations of these stressors in order to evaluate their cumulative effects on freshwater ecosystems at different scales of biological organization (community, ecosystem, meta-ecosystem). First, I evaluated stream invertebrate community responses to sedimentation, nutrient enrichment, and the insecticide chlorpyrifos using laboratory microcosms with distinct microhabitats. I demonstrated that chlorpyrifos can interact non-additively with fine sediment (reversal) and nutrients (antagonism), with potentially deleterious impacts on small-sized invertebrates. Furthermore, invertebrates in gravel microhabitats were more severely affected than those in leaf packs. Second, I manipulated levels of nutrients, sediment, and the insecticide imidacloprid in experimental pond ecosystems. I demonstrated these stressors had antagonistic effects on pelagic and benthic invertebrate diversity. Moreover, the results suggested imidacloprid increased ecosystem metabolism indirectly, through negative effects on invertebrate consumers. Finally, I explored processes at the scale of the river network meta-ecosystem. Using a network of experimental channels, I investigated how multiple-stressor interactions within tributaries affected downstream ecosystems. My results indicated that complex nutrient-sediment interactions within tributaries could strongly alter the flux of organisms from tributaries to downstream ecosystems. Furthermore, I observed that at small spatial scales, these alterations of within-network migration patterns could be more influential than the transport of the stressors from headwaters to recipient ecosystems. My
research contributes novel evidence suggesting that complex interactions among nutrient enrichment, sedimentation, and insecticide contamination are problematic in freshwater ecosystems, and have distinct mechanisms operating at different scales. In particular, these findings underscore the importance of considering multiple-stressor interactions in insecticide environmental risk assessments; even at low concentrations, interactions with other stressors may result in unexpected negative effects for aquatic biota and ecosystem processes.
Lay Summary

Agriculture is a global driver of degradation of freshwater ecosystems, affecting them through discharges of nutrients, sediment, and insecticides. Although these disturbances often occur simultaneously, little is known about their potential to interact synergistically (i.e., intensify each other’s effects), which creates uncertainties in the prediction of their combined environmental impacts. To address this research gap, I evaluated individual and combined effects of nutrients, sediment, and insecticides on experimental freshwater ecosystems. I found that insecticide toxicity could be enhanced or mitigated by nutrient and sediment additions, with the outcome depending on the characteristics of the system and the insecticide. Moreover, in river networks synergistic interactions within tributaries had surprising impacts on recipient ecosystems downstream. My results suggest that complex interactions among simultaneous disturbances are frequent and may have unexpected, negative effects on aquatic life. Consideration of these potential interactions is important to protect freshwater ecosystems in agricultural landscapes.
Preface

Chapter 2: Chlorpyrifos interacts with other agricultural stressors to alter stream communities in laboratory microcosms
Authors: AM Chará-Serna, JS Richardson
Status: Accepted 19/09/2017
Journal: Ecological Applications (in press)
Comments: This study was conceptualized by AMCS and JSR. AMCS conducted the experiment, collected the data, and performed laboratory work. AMCS analyzed the data and wrote the paper with input from JSR.

Chapter 3: Nutrients and sediment modify the impacts of a neonicotinoid insecticide in experimental pond ecosystems
Authors: AM Chará-Serna, LE, Christy A. Morrissey, JS Richardson
Status: In preparation (anticipated submission date: 15/1/2018)
Comments: This study was conceptualized by AMCS and JSR. AMCS and JSR prepared the mesocosms and experimental set-up. AMCS and LE conducted the experiment, collected the data, and performed laboratory work. CAM analyzed insecticide water samples. AMCS analyzed the data and wrote the paper with input from JSR. LE and CAM provided edits on the manuscript.

Chapter 4: Multiple-stressor interactions in headwater streams and their impacts to downstream ecosystems
Authors: AM Chará-Serna, JS Richardson
Status: In preparation (anticipated submission date: 27/2/2018)
Comments: This study was conceptualized by AMCS and JSR. AMCS conducted
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Thank you for being the best ropemate I could ever ask for.
Chapter 1

Introduction

1.1 Conservation of freshwater ecosystems

Freshwater ecosystems are the most threatened ecosystems worldwide (Saunders et al., 2002; Dudgeon et al., 2006; Abell et al., 2007; Nel et al., 2009; Strayer and Dudgeon, 2010; Vörösmarty et al., 2010). Due to the accelerated growth of human population and economy over the past century, the use of fresh waters has increased rapidly, causing massive negative impacts on these ecosystems (Strayer and Dudgeon, 2010). Threats to fresh waters have been extensively documented, with habitat degradation and loss, water pollution, flow modification, land use change, over-exploitation, and introduction of non-native species, figuring as the most important drivers of the declining trends (Dudgeon et al., 2006; Allan and Castillo, 2007; Dyke, 2008). As of 2010, approximately 65% of the continental water discharge was moderately to highly threatened by human activities, with 30 of the world’s 47 largest rivers showing moderate to high incidence of threats (Vörösmarty et al., 2010). These levels of degradation have resulted in alarming declines in freshwater biodiversity (Dudgeon et al., 2006; Strayer and Dudgeon, 2010), including the loss or imperilment of over 10,000 species (Strayer and Dudgeon, 2010). Moreover, the degradation of fresh waters has direct impacts on human populations, who depend on water as their most essential natural resource. According to a recent global spa-
tial analysis, 80% of the world’s population lives in areas with low water security, which means that about 4.8 billion people do not have reliable access to potable water (Vörösmarty et al., 2010).

Freshwater ecosystems have a high interface with the landscape, which makes them extremely vulnerable to land-use alterations (Allan and Johnson, 1997, Allan, 2004). Anthropogenic land use affects these ecosystems through different processes that operate at multiple spatial scales (Allan and Johnson, 1997). These processes include alterations of habitat structure and organic matter inputs, which are primarily governed by local conditions, and alterations to nutrient supply, sediment inputs, hydrology, and geomorphology, that are mainly influenced by conditions at the regional scale (Allan and Johnson, 1997). Furthermore, it has been long recognized that human disturbances on fresh waters rarely occur in isolation, and that certain land uses, such as agriculture and urbanization, tend to cause multiple, simultaneous impacts on these ecosystems (Allan, 2004; Townsend et al., 2008). However, to date we still lack a predictive understanding of the mechanisms by which these impacts alter ecological function and structure of fresh waters.

1.2 Impacts of agriculture on freshwater ecosystems

Agriculture is the most important modifier of water-related ecosystem services around the globe (MEA, 2005; Vörösmarty et al., 2010; Smukler et al., 2012). Currently more than 15% of the global precipitation runs through cultivated landscapes, and up to 70% of the global water withdrawals are used for agricultural activities (Smukler et al., 2012). As a result, agriculture is the main non-point source of excess nutrients, fine sediment, and toxic and organic pollutants to aquatic ecosystems worldwide (Allan, 2004; MEA, 2005; Vörösmarty et al., 2010). Furthermore, agricultural practices often result in degradation or loss of riparian forests, which in turn alters organic matter inputs, hydrology, flow regimes, and channel morphology of natural water bodies (Allan, 2004; Johnson and Host, 2010). All these physical and chemical modifications ultimately impair habitat quality and alter resource availability for biological communities, causing shifts in their trophic structure and composition (Allan, 2004; Diana et al., 2006; Johnson and
The nutrient pollution caused by the use of fertilizers in agricultural landscapes has long been recognized as a global threat to freshwater and marine ecosystems (Vörösmarty et al., 2010; Woodward et al., 2012b; Rosemond et al., 2015). Nutrient enrichment has been linked to alterations in allochthonous and autochthonous basal resources of freshwater food webs (Woodward et al., 2012b; Rosemond et al., 2015). At low levels, nutrient inputs stimulate algae growth and accelerate leaf decomposition due to increased microbial processing (Rosemond et al., 2015; García et al., 2017). Therefore, even though moderate nutrient inputs cause bottom-up effects for the autochthonous component of freshwater food webs, they are also associated with increased mineralization of organic carbon. This in turns results in release of CO₂ and lower energy transfers to higher trophic levels for the allochthonous component of such food webs (Rosemond et al., 2015; Atwood et al., 2015). Furthermore, high levels of nutrient enrichment can be toxic to aquatic organisms and are associated with hazardous algal blooms, and dissolved oxygen depletion in running waters and hypoxia in lentic and coastal ecosystems (Allan, 2004; Riseng et al., 2011).

Agricultural practices are also associated with increased fine sediment runoff due to the replacement of the natural vegetation cover and livestock trampling (Allan, 2004). The term fine sediment is used henceforth to describe sediment with particles of less than 2 mm in size (Wood and Armitage, 1997), thus including very coarse sand (1000 – 2000 µm), coarse sand (500 – 1000 µm), medium sand (250 – 500 µm), fine sand (125 – 250 µm), very fine sand (62 – 125 µm), silt (4 – 62 µm), and clay (< 4 µm), according to the Wentworth scale (Wentworth, 1922). Increased loads of fine sediment are documented to have particularly harmful impacts on freshwater ecosystems (Waters, 1995; Wood and Armitage, 1997; Allan, 2004). Several recent experimental manipulations have observed that compared to other agricultural impacts, such as nutrient enrichment, warming, and glyphosate contamination, sedimentation had the most detrimental effects on invertebrate communities and ecological function (Piggott et al., 2012; Magbanua et al., 2013; Piggott et al., 2015b). The entrance of fine sediment into freshwaters increases water column turbidity, limiting light penetration and reduc-
ing primary production, with consequent negative impacts on higher trophic levels (Wood and Armitage, 1997; Wagenhoff et al., 2013). Once deposited, fine sediment can greatly impair habitat quality for aquatic organisms, smothering periphyton and biofilm, and clogging interstitial spaces that are refugia for invertebrates and gravel-spawning fish (Allan, 2004; Matthaei et al., 2006; Louhi et al., 2011). Furthermore, fine sediment is reported to induce invertebrate drift, and cover gills and respiratory surfaces with deleterious effects on sensitive organisms (Allan, 2004; Piggott et al., 2015b).

The widespread use of pesticides in agricultural catchments often results in contamination of freshwater ecosystems, usually through spray drift or runoff (Allan, 2004; Sánchez-Bayo, 2011; Morrissey et al., 2015). Even though it has been long recognized that pesticides may play a big role in ecological degradation, the concentration of agricultural pesticides is rarely measured in studies relating agricultural land use and freshwater biota (Allan, 2004; Stehle and Schulz, 2015; Schäfer et al., 2016; Gessner and Tlili, 2016). However, recent global estimations indicate that pesticides are among some of the world’s predominant sources of freshwater pollution (Vörösmarty et al., 2010; Schäfer et al., 2016), with continental- and country-scale surveys in Europe, North America, and Australia reporting frequent detection of pesticides at concentrations that exceed regulatory risk thresholds (Gilliom, 2007; Malaj et al., 2014; Stehle and Schulz, 2015; Morrissey et al., 2015; Schäfer et al., 2016; Sánchez-Bayo et al., 2016). The impacts of pesticides on freshwater ecosystems are diverse and largely depend on the mode of action of their active ingredients, their target organisms, and the magnitude and duration of exposures (Sánchez-Bayo, 2011). However, some recent large-scale surveys indicate that in general, pesticide contamination in freshwater ecosystems is associated with significant losses of fish and invertebrate diversity, as well as alterations in important ecosystem processes (Schäfer et al., 2012; Beketov et al., 2013; Malaj et al., 2014). Moreover, some of these studies registered such effects at concentrations that are considered safe by European Union regulations, highlighting the urgent need to review current methodologies for pesticide risk assessment (Schäfer et al., 2012; Beketov et al., 2013).
1.3 Cumulative effects

Anthropogenic stressors are physical, chemical, and biological factors that, as a result of human activities, exceed their natural ranges of variation, causing measurable biological and ecological responses (Townsend et al., 2008; Crain et al., 2008; Statzner and Bêche, 2010). The changes an ecosystem experiences due to the combination of multiple stressors are herein denominated cumulative effects (Macdonald, 2000; Crain et al., 2008; Seitz et al., 2011). Researchers have long been interested in cumulative effects because they are often difficult to predict on the basis of single stressor responses. Increasing evidence highlights the importance of complex interactions, in which the combination of two or more stressors results in negative effects that are amplified (synergistic interaction), mitigated (antagonistic interaction), or reversed (reversal interaction), relative to what is expected from the stressors’ individual effects (Folt et al., 1999; Townsend et al., 2008; Matthaei et al., 2010; Piggott et al., 2015c). For example, in their highly cited meta-analysis of cumulative effects on marine ecosystems, Crain et al. (2008) found that the overall interaction effect across all studies was synergistic. Similarly, Holmstrup et al. (2010) found that synergistic interactions among natural stressors and toxicants were common in 150 animal-focused studies. Moreover, a recent meta-analysis of experimental studies in freshwater ecosystems reported that 41% of the multiple-stressor interactions were antagonistic, while 28% were synergistic (Jackson et al., 2016).

Synergistic interactions generally take place when the biological response to one or more of the stressors involved is non-linear (e.g. a certain impact has a disproportionate effect on the community), or when a combination of activities triggers secondary effects that enhance their impact on the ecosystem (Macdonald, 2000; Crain et al., 2008; Seitz et al., 2011). On the other hand, antagonistic interactions are observed when the influence of one stressor dampens the impact of a second stressor (Folt et al., 1999; Crain et al., 2008). The importance of understanding and predicting these phenomena was highlighted almost two decades ago (Breitburg et al., 1998; Folt et al., 1999), and is still considered one of the most pressing issues in ecology and conservation (Wagenhoff et al., 2013; Piggott
et al., 2015b; Jackson et al., 2016). Distinguishing categories of multiple-stressor interactions is fundamental to improve our understanding of their mechanisms, ultimately improving our ability to predict cumulative effects (Folt et al., 1999; Crain et al., 2008). Furthermore, disentangling multiple-stressor effects allows us to classify stressors based on severity, providing us with strategies to better manage and mitigate their impacts on natural ecosystems (Townsend et al., 2008; Statzner and Bèche, 2010).

1.3.1 Conceptual models to interpret cumulative effects

Even though the terms “synergistic” and “antagonistic” have been used extensively in the scientific literature, their mathematical definitions vary according to different conceptual frameworks. Folt et al. (1999) highlighted this lack of consensus and described three broad conceptual models to categorize multiple stressor effects: the comparative effects model, the additive model, and the multiplicative model.

**Comparative model:** This model is appropriate when the combined effect of two stressors is equal to the effect of the single worst stressor (Folt et al., 1999). This model is useful in systems where a single stressor is the main driver of degradation. Once this dominant stressor affects a system, other lesser stressors have no additional impact. In this model, combined effects that are greater or less than the effect of the dominant stressor are synergistic or antagonistic, respectively (Folt et al., 1999).

**Additive model:** This is the most commonly used model in theoretical and applied research on multiple stressors (Folt et al., 1999; Crain et al., 2008; Dunne, 2009; Piggott et al., 2015c). In this framework, the combined effect of multiple stressors is assumed to equal the sum of the individual stressor effects (Reid, 1993; Crain et al., 2008). Deviations that result in effects that are greater or less than the sum of the individual effects are considered synergistic and antagonistic, respectively. Despite its widespread use, some authors argue that this model cannot detect synergistic interactions when the sum of the individual effects exceeds 100% (Pennings, 1996; Folt et al., 1999), which limits its usefulness to evaluate interactions...
when individual stressor effects are high.

**Multiplicative model:** This model assumes that the combined effects of multiple stressors are equal to the product of the individual effects. Thus, antagonistic and synergistic effects are less or more than that expected from the multiplicative interactions of the stressors (Folt et al., 1999; Townsend et al., 2008).

Because these three models mathematically result in different interpretations of interactions between cumulative stressors, Folt et al. (1999) emphasized the importance of specifying which model is used when conducting cumulative effects research. Furthermore, after carrying out an empirical test comparing the applicability of the three models, they concluded that no single best model can be recommended for cumulative effects research. Instead, each researcher should choose the proper framework depending on the stress mechanisms of the system under study, and explicitly state why it was chosen.

1.4 Cumulative effects in freshwater ecology

The issue of cumulative effects in freshwater ecosystems has received increasing attention in the past few decades. Initially, with the recognition of the multi-stressor template in which freshwater ecosystems function, a number of studies evaluated the response of freshwater communities and habitats to multiple stressors acting simultaneously (e.g., Swank and Bolstad, 1994; Greathouse et al., 2005; Alexander and Culp, 2008; Dodds and Oakes, 2008; Aristi et al., 2012). Following this effort, freshwater research took a step forward to measure the relative effect of individual stressors, as a means to distinguish the most important drivers of degradation (Comte et al., 2010; Riseng et al., 2010; Esselman and Allan, 2010; Wepener et al., 2011; Damásio et al., 2011; Wooster et al., 2012). Along with these studies, cumulative effects’ implications on management issues have received a lot of attention, with papers proposing methodologies to evaluate cumulative effects (e.g., Chen, 1992; Bevenger and King, 1995; Smit and Spanling, 1995; Loftis et al., 2001; Johnson et al., 2012), and others discussing their incorporation into management and environmental policies (e.g., Macdonald, 2000; Cooper and Sheate, 2002; Noble, 2002).
Even though the implications of cumulative effects are already being considered in management frameworks, there is still much to learn empirically about complex interactions of some widespread ecological stressors (Nöges et al., 2016). Only in the past couple of decades have researchers begun implementing field and experimental studies with the specific purpose of measuring the nature of the interactions among different anthropogenic stressors (e.g., Wagner et al., 1997; Townsend et al., 2008; Kratina et al., 2012; Clements et al., 2013; Piggott et al., 2015a; Alexander et al., 2016). In a recent meta-analysis, Jackson et al. (2016) identified 88 papers examining multiple-stressor interactions on freshwater ecosystem receptors. These studies span the years 1995 to 2014, and have addressed different stressor combinations (e.g., pH, UV radiation, warming, invasive species, ammonia, nutrients, fine sediment, heavy metals, water abstraction, predators, and pesticides), at different scales of biological organization, ranging from unicellular organisms to whole ecosystems (Jackson et al., 2016). These investigations have not only documented previously unknown cause-and-effect relationships between stressors and biological responses, but have also underscored the prevalence of complex multiple-stressor interactions in freshwater ecosystems. According to Jackson et al. (2016), 84% of the interactions analyzed in those studies were non-additive, with antagonistic interactions representing 41%, followed by synergistic (28%), and reversal interactions (15%). These findings are particularly relevant to environmental management, as they suggest that additive environmental risk assessment frameworks may underestimate or overestimate cumulative effects of common stressors on freshwater ecosystems.

Despite these important advances, some stressors and ecosystems have been not been sufficiently studied under the cumulative effects perspective. For instance, only 7 out of the 88 studies analyzed by Jackson et al. (2016) addressed interactive effects of pesticides. Understanding how the presence of additional stressors may affect the impacts of pesticides is a critical research need, especially after a recent investigation found that environmental stressors could increase individual toxicity to pesticides by a factor up to 100 (Liess et al., 2016). This issue is particularly important for wetlands and shallow ponds in agricultural landscapes (Roessink et al., 2010; Seitz et al., 2011; Noble et al., 2011; Sheelanere et al., 2013).
These lentic environments, provide important ecosystem services to agricultural production, and are habitat to waterbirds, amphibians, and invertebrates (Main et al., 2014; Dodds and Whiles, 2010). Yet, they are frequently exposed to pesticide contamination, eutrophication, and sedimentation (Main et al., 2014; Skagen et al., 2008). Moreover, compared to other freshwater ecosystems, their hydraulic characteristics favour sediment deposition and long residence times (Luo et al., 1997; Skagen et al., 2008; Dodds and Whiles, 2010). Both conditions can potentially increase the exposure of organisms to toxicants through longer persistence of the chemical in the water column, contact with contaminated with bed sediment, and resuspension of sediment particles (Warren et al., 2003; Roessink et al., 2008; Burton and Johnston, 2010). Research is needed to understand if the combination of these factors ultimately enhances the detrimental impacts of pesticides on these important ecosystems.

Studying cumulative effects on freshwater ecosystems becomes increasingly challenging at larger spatial scales (Loftis et al., 2001). For example, at the scale of river networks cumulative watershed effects (i.e. changes that involve watershed processes and are influenced by multiple stressors) are important (Reid, 1993; Freeman et al., 2007). Yet, there are few empirical studies addressing the subject of cumulative effects from the river network perspective (but see Tomscha et al., 2017). Thus, few evaluations of how disturbances in multiple headwater systems are transmitted down the river network, how complex interactions among stressors in headwater ecosystems affect ecosystems downstream, or how the loss of headwaters affects river network function and resiliency (Freeman et al., 2007). Research to answer these type of questions is challenging as it may involve spatial and temporal lags in system responses, dilution of impacts, geographic decoupling between cause and effect, and site-specific variations in impact expression (Reid, 1993, 1998; Gomi et al., 2002). However, it is fundamental to understand mechanisms of disturbance at the scale of river networks and formulate adequate strategies of prevention and mitigation.
1.5 Predicting ecosystem response to cumulative stressors

Predicting cumulative effects is extremely challenging due to the high complexity of the systems involved. The response of natural systems to multiple stressors is determined by characteristics of both the ecosystem and the stressors at play (Breitburg et al., 1998; Vinebrooke et al., 2004). Some ecosystem characteristics that modulate response to multiple stressors include: species diversity (especially the number of redundant species); openness of the system (increases ability to recover from disturbance); temporal variability (i.e. successional processes); spatial patterns; and ecological and evolutionary history (Breitburg et al., 1998; Crain et al., 2008; Statzner and Bèche, 2010). On the other hand, stressors’ specificity, their modes of action, their potential for interaction, the magnitude of their impacts, and temporal patterns of occurrence (frequency or duration of the exposition, simultaneous versus consecutive occurrence of stressors), are some stressor characteristics that will influence the outcome of the disturbance (Breitburg et al., 1998; Crain et al., 2008).

Certain circumstances will result in cumulative effects that are easier to predict than others. For example, it is reasonable to expect an inverse relationship between the magnitude of the response to multiple stressors and the number of redundant species in the system (Breitburg et al., 1998). Diverse systems with many functionally similar species may be more stable due to differences in species’ tolerance to stressors that promote complementary responses, i.e. tolerant species increase as sensitive species decline (Breitburg et al., 1998). In this case, an increase in the number of stressors with independent modes of action, means that fewer tolerant species will be able to survive and benefit from the disturbance (Breitburg et al., 1998; Statzner and Bèche, 2010). However, the relationship between the magnitude of the response and the number of stressors will change in communities with strongly dominant or keystone species. In the latter case, response to multiple stressors may be similar to the response to a single stressor, if that single stressor affects the dominant or keystone species (Breitburg et al., 1998). Another case when the system may respond similarly to a single stressor as to multiple stressors,
is when the stressors have similar modes of action. Once the system responds to one stressor (e.g., by losing species sensitive to that stressor) no further losses will be observed if the second stressor targets the same type of organisms, through the same mechanisms (Breitburg et al., 1998).

On the other hand, the relationship between functional redundancy and stability of processes at the ecosystem level is much less clear when the modes of action of the different stressors are not independent. In the common case when the action of one stressor affects mechanistically or statistically the action of a second stressor, is when we observe “ecological surprises” with unexpected antagonistic or synergistic effects (Breitburg et al., 1998). The cases illustrated above portray only a dimension of the complexity involved in the prediction of multiple effects. However challenging, enough empirical information would eventually allow for reasonable predictions of when and where certain interactions are expected to occur, improving our ability to manage their impacts.

1.6 Thesis objectives and overview

Sedimentation, nutrient enrichment, and insecticide pollution are some of the most pervasive freshwater ecosystem stressors associated with agricultural activities worldwide (MEA, 2005; Vorösmarty et al., 2010). However, despite their obvious co-occurrence and the potential for complex interactions among them, there is relatively little empirical information about their pairwise combinations and virtually no information about their potential three-way interactions. The overarching objective of my thesis was to improve our mechanistic understanding of the individual and cumulative impacts of these three stressors on freshwater ecosystems. Thus, I experimentally manipulated levels of sediment, nutrients, and insecticide contamination on mesocosms recreating different types of freshwater ecosystems, and tested hypotheses about impacts operating at different scales of biological organization. My experiments included: a stream microcosm manipulation to test effects at the scale of communities (Chapter 2), a pond mesocosm manipulation to test effects at the scale of ecosystems (Chapter 3), and a stream mesocosm network manipulation to test effects at the scale of river meta-ecosystems (Chapter 4).
In Chapter 2, I evaluated in detail how benthic invertebrate communities responded to low concentrations of chlorpyrifos when applied in combination with sedimentation and nutrient enrichment. Chlorpyrifos is a widely used organophosphorus insecticide that is generally considered safe for non-target organisms. However, to date its effects have not been evaluated in the context of other common agricultural stressors. Using a fully-crossed factorial experiment in laboratory microcosms, I tested the hypothesis that chlorpyrifos would interact non-additively with nutrient and sediment additions in two distinct microhabitats (leaf packs and gravel), and evaluated short-term indirect effects of the insecticide on two important ecosystem processes (leaf decomposition and primary production).

In Chapter 3, I further explored potential non-additive interactions among agricultural stressors by testing cumulative impacts of imidacloprid, nutrient enrichment, and sedimentation on shallow pond ecosystems. Imidacloprid, a systemic insecticide from the family of neonicotinoids, currently ranks amongst the most widely used agricultural insecticides in the world. Despite its popularity, relatively little is known about its potential to interact with other agricultural stressors and alter freshwater ecosystem functioning. I used a fully crossed factorial manipulation in outdoor pond mesocosms to test the hypothesis that imidacloprid would interact non-additively with nutrient and fine sediment additions to alter benthic and planktonic communities. Further, I tested whether structural impacts on pelagic and benthic compartments of pond ecosystems would ultimately translate into ecosystem-wide alterations on metabolism and organic matter processing.

In my final data chapter (Chapter 4), I used a network of outdoor stream mesocosm channels to investigate potential consequences of multiple-stressor interactions at the scale of river networks. I manipulated sediment and nutrient levels in the tributaries of second-order channels, to determine individual and combined effects of the stressors at the tributaries and recipient channels. Specifically, I tested the hypothesis that complex-multiple stressor interactions within tributaries would influence responses (benthic invertebrate density, invertebrate drift, and leaf decomposition) in downstream channels, and that increasing levels of disturbance in the tributaries would cause proportional increases of disturbance on downstream mesocosms.
Chapter 2

Chlorpyrifos interacts with other agricultural stressors to alter stream communities in laboratory microcosms

2.1 Introduction

Over the past decades, agriculture has become one of the most important drivers of freshwater ecosystem degradation around the world (MEA, 2005; Smukler et al., 2012). Agricultural lands currently cover about 40% of the ice-free land surface (Ramankutty et al., 2010), receive more than 15% of the global precipitation, and are responsible for more than 70% of the water withdrawals (Smukler et al., 2012). The impacts of agriculture on freshwaters are context dependent and involve multiple ecological stressors interacting in space and time (Allan, 2004; Matthaei et al., 2010; Riseng et al., 2011; Chará-Serna et al., 2015). The effects of some of these ecological stressors have been extensively documented. For example, it is well established that many agricultural practices increase inputs of fine sediment into wa-
ter bodies, generally causing negative effects on invertebrate communities and ecological processes (Wood and Armitage, 1997; Allan, 2004; Benoy et al., 2012; Burdon et al., 2013). It is also widely acknowledged that the application of fertilizers increases the input of nutrients into freshwater food webs, causing subsidy-stress responses in biological communities and ecological function (Woodward et al., 2012a; Rosemond et al., 2015; Richardson and Wipfli, 2016). However, agricultural stressors rarely operate in isolation, and their cumulative effects are less well understood. In the past decades, the potential complex interactions among agricultural stressors have received increasing attention, with several experimental studies evaluating 2-way and 3-way combinations of stressors like fine sediment, nutrient enrichment, water abstraction, warming and glyphosate (e.g., Matthaei et al., 2010; Wagenhoff et al., 2012; Magbanua et al., 2013). Nevertheless, some pervasive agricultural stressors, like insecticide contamination, have received relatively less attention in the context of other anthropogenic stressors.

The organophosphorus insecticide chlorpyrifos perfectly illustrates this situation. Chlorpyrifos is one of the most widely applied insecticides in the world (Gebremariam, 2011; Giesy and Solomon, 2014). In the United States alone, about 3.2 to 4.1 million kilograms of chlorpyrifos were applied per year in the last decade, for the control of insect pests and mites (Solomon et al., 2014). Not surprisingly, chlorpyrifos is frequently detected in agricultural water bodies around the world in concentrations that sometimes exceed aquatic-life-protection criteria (Phillips and Bode, 2004; Marino and Ronco, 2005; Williams et al., 2014). As a broad spectrum neurotoxic insecticide, chlorpyrifos is highly toxic to non-target aquatic invertebrates and, to a lesser extent, to vertebrates (Giesy et al., 1999). Hence, a number of experimental and field studies have been conducted to evaluate its effects on aquatic organisms and ecosystem processes (e.g., Brock et al., 1992b; Pusey et al., 1994; Daam et al., 2008). However, relatively few empirical studies have addressed potential complex interactions of the insecticide with other agricultural stressors (but see Cuppen et al., 2002; Traas et al., 2004; Alexander et al., 2013), and to our knowledge no studies have explicitly tested three-way interactions between chlorpyrifos, nutrient enrichment, and sedimentation.

There are several plausible mechanisms by which the negative impacts of chlor-
pyrifos could be enhanced (synergistic interaction) or mitigated (antagonistic interaction) by sedimentation and nutrient enrichment. For instance, chlorpyrifos is moderately hydrophobic, so it tends to partition from the aqueous phase to be strongly adsorbed by the sediment when it enters water bodies (Giesy et al., 1999; Gebremariam, 2011). This tendency to concentrate in the sediment may result in enhanced exposure of invertebrates in streams that are simultaneously affected by sedimentation, through contact and ingestion of contaminated sediment particles. Furthermore, a previous evaluation of interactions among an insecticide mixture that contained chlorpyrifos, and different levels of nutrient enrichment, observed that moderate levels of eutrophication mitigated the negative effects of the insecticide mixture, whereas high levels of eutrophication enhanced them (Alexander et al., 2013).

Here, we describe the results of a community-level, microcosm experiment designed to evaluate individual and cumulative effects of chlorpyrifos, sedimentation, and nutrient enrichment on stream invertebrate communities (abundance, biomass, richness, size structure, composition) and ecosystem processes (primary productivity and organic matter decomposition), at the scale of the microhabitat. We were particularly interested in testing potential non-additive interactions among the three agricultural stressors, and evaluating short-term effects of the insecticide on ecosystem processes. Based on findings of previous investigations, we predicted that at the stressor levels tested in our study, we would observe: 1) positive individual effects of nutrients on periphyton biomass, leaf decomposition, and invertebrate biomass; 2) negative individual effects of sedimentation on most invertebrate community metrics and ecosystem processes; 3) negative individual effects of chlorpyrifos on invertebrate abundance, richness, and biomass; 4) indirect negative effects of chlorpyrifos on leaf decomposition (through the inhibition of shredders); 5) indirect positive effects of chlorpyrifos on periphyton biomass (through the inhibition of grazers); 6) synergistic interactions between chlorpyrifos and sedimentation enhancing negative impacts on invertebrate communities; and 7) antagonistic interactions between chlorpyrifos and nutrient enrichment mitigating negative effects on invertebrate communities.
2.2 Methods

2.2.1 Experimental design

We conducted a 15-day factorial manipulation of sedimentation, nutrient enrichment, and chlorpyrifos contamination in 32 laboratory microcosms, using two levels of each stressor (presence, absence) in a fully crossed factorial design with eight treatments and four replicates of each treatment (Figure 2.1). Each microcosm consisted of a 38 L aquarium (25.4 x 50.8 x 30.5 cm) filled with 12 L of stream water. We modified the design used by Sanpera-Calbet et al. (2012), placing a 25 x 30 cm glass sheet parallel to the tank bottom to partially divide the tank into an upper and a lower section. We then installed a bubbler connected to an air pump at the bottom of one end of the tank, with a plastic deflector above to generate visible circular flow in the tanks (see Appendix Figure A.1 for a schematic). The lower section of each tank was stocked with 2 L of washed mixed gravels (0.5-2 cm grain size range), arranged for a mean substrate depth of 1.5 cm. Stream water was collected from Spring Creek, a relatively non-perturbed, third-order stream located in the Malcolm Knapp Research Forest (British Columbia, Canada; 49° 16′ N, 122° 34′ W), that also served as a source for aquatic invertebrates. This stream is characterized by high dissolved oxygen concentrations (near saturation), low suspended solids (0.4-2.2 mg L\(^{-1}\)), and neutral to slightly acidic water (pH: 6.37-6.73). The microcosms were situated in a laboratory at the University of British Columbia under similar light (ViaV olt T5 high output fluorescent grow lights) and temperature (water temperature 20 ± 0.15°C) conditions, and had similar values for dissolved oxygen (9.0 ± 0.04 mg O\(_2\) L\(^{-1}\)), pH (7.4 ± 0.02), and conductivity (0.02 ± 0.01 mS cm\(^{-1}\)) before the application of the treatments.

Microcosms were inoculated with invertebrate densities 15% higher than densities found in Spring Creek, in order to offset for mortality due to transport to the laboratory and manipulation. For every three microcosms, five Surber samples (Surber area = 0.09 m, mesh size = 500 µm) were collected, pooled, and subsampled into three equal portions. This procedure was repeated 11 times to obtain 32 replicate samples that were randomly assigned to microcosms. Once inoculated
with aquatic invertebrates, and after deploying leaf packs and ceramic tiles (see below), the microcosms were allowed to equilibrate for one day before the application of treatments. Laboratory microcosms inoculated with natural invertebrate communities cannot incorporate all the complex dynamics of natural ecosystems but they provide a useful tool to study short-term community responses to controlled manipulations of multiple stressors. This approach offers sufficient replication for hypothesis testing and has been successfully employed in the past in a number of studies on invertebrate ecology and toxicology (e.g., Kiffney and Clements 1996b; Clements et al. 2013).

2.2.2 Stressor treatments

Stressor treatments were randomly assigned to the microcosms and applied once on day 1 of the experiment. For the sediment treatment, 0.8 L of sand (0.25 mm mean grain size, “fine sand” according to the Wentworth scale) were added as evenly as possible to each sediment addition microcosm, resulting in 60 ± 2% (mean ± Standard Error [SE], visual estimation) of sediment coverage by streambed area and 8.5 ± 2.1 mm (mean ± SE, measured with a ruler) of sediment deposited on top of the original gravel substrate. These sediment additions are equivalent to values reported in rivers affected by farming practices (Townsend et al. 2008; Waggonhoff et al. 2012), and are similar to those used in several previous experimental assessments of the effects of sedimentation in stream ecosystems (e.g., Matthaei et al. 2010; Piggott et al. 2015a; Louhi et al. 2017).

Background nutrient concentrations (NH₄-N: 19.8 ± 0.6 µg L⁻¹, NO₃-N: 60 ± 11.4 µg L⁻¹, PO₄-P: 2.5 ± 0.1 µg L⁻¹, mean ± SE) were augmented in nutrient addition tanks using potassium phosphate (KH₂PO₄: 83 µg L⁻¹) and ammonium nitrate (NH₄NO₃: 155 µg L⁻¹). Analytical grade chlorpyrifos (>100%, Pestanal® Sigma-Aldrich) was used for the insecticide treatment. A stock solution of the insecticide was prepared in 99.5% analytical grade ethanol and applied as a single pulse to each treated tank using a micropipette for a nominal chlorpyrifos concentration of 0.3 µg L⁻¹. Target chlorpyrifos concentration falls within the range of chlorpyrifos concentrations that have been frequently reported in surface waters of
agricultural landscapes in countries like the United States (Kimbrough and Litke, 1996; Williams et al., 2014) and Argentina (Jergentz et al., 2005). A clean micropipette was used to apply ethanol to all non-insecticide treatment tanks to control for potential solvent effects. The volume of ethanol applied in non-insecticide tanks was the same as the volume added with the insecticide application (400 µL).

### 2.2.3 Response parameters

Water samples collected 36 hours after treatment additions were used to determine chlorpyrifos concentrations using standard GC/MS methods (Price et al., 2009), subsequent water sampling three days after treatment application was conducted to determine suspended solids, PO$_4$-P, NH$_4$-N, and NO$_3$-N concentrations (APHA, 2005; Hauer and Lamberti, 2007). Measures of dissolved oxygen concentration, conductivity, and pH were recorded weekly after the application of treatments using hand-held probes.

All the gravel from each microcosm was sampled for invertebrates on day 15. We washed the gravel in a 2 mm sieve stacked on top of a 250 µm sieve. Gravel retained in the 2 mm sieve was sorted for invertebrates at the time of collection, and the material retained in the 250 µm sieve was stored in 80% ethanol and sorted later under the dissecting microscope. Half-decomposed invertebrates showing clear indications of being dead before the day of sample collection were discarded, remaining individuals were enumerated for each microhabitat (leaf packs and gravel), identified to the lowest practical taxonomic level (usually genus), and measured to determine dry mass from length-mass regressions (Smock, 1980; Benke et al., 1999; Johnston and Cunjak, 1999). Given the characteristics of our indoor microcosms and the short duration of the experiment, we can assume the experimental invertebrate communities were closed, with no reproduction, immigration or emigration processes affecting invertebrate density (e.g. we did not observe insect emergence during the experiment). Thus, the reductions in density documented by the end of the experiment likely reflect invertebrate mortality due to the treatments.
One day before treatment application (day 0), a coarse-mesh alder leaf pack (Alnus rubra, 1 g air-dry weight) was introduced to each microcosm to measure leaf decomposition. Leaves used for this study were collected after abscission in the Malcolm Knapp Research Forest. On day 15, leaf packs were removed from the microcosms, and stored at -18°C until processing. Posterior processing involved defrosting and rinsing over a 250 µm sieve that was sorted for invertebrates. The remaining leaf material was dried at 60°C for 5 days, weighed, ashed at 500°C, and reweighed to calculate ash-free dry mass.

Unglazed, 7 cm² ceramic tiles were used to measure periphyton biomass in the microcosms. Prior the beginning of the experiment, the tiles were incubated for 10 days in Spring Creek to promote algae colonization. Conditioned tiles were refrigerated and transported to the laboratory, where one tile was introduced into each microcosm on day 0. On day 15, tiles were removed from the microcosms and stored in the darkness at -18°C for later processing. Periphyton biomass was estimated as Chlorophyll-a using standard fluorometric methods (Arar and Collins, 1997).

2.2.4 Data analysis

We computed a total of 20 response variables, including two measures of ecosystem processes (leaf decomposition and periphyton biomass), and nine measures of community structure that were calculated separately for invertebrates collected in gravel and invertebrates collected in leaf packs (total invertebrate density, total invertebrate biomass, total invertebrate richness, abundance of Ephemeroptera, Plecoptera, and Trichoptera [EPT] taxa, biomass of EPT taxa, richness of EPT taxa, average body size, abundance of small [< 5 mm] individuals, and abundance of large [> 5 mm] individuals). We used three-way, factorial ANOVAs to evaluate the individual and combined effects of nutrient enrichment (N), sedimentation (S), and chlorpyrifos insecticide (I) on these univariate responses. For each response variable the linear model tested was: $y = b_0 + b_1N + b_2S + b_3I + b_4NS + b_5NI + b_6SI + b_7NSI$, where $N$ is the nutrient treatment, $S$ the sediment treatment, and $I$ the insecticide treatment. Because two invertebrate samples were accidentally lost
during collection and processing, we used type-III sum of squares, which is robust to unbalanced designs (Quinn and Keough, 2002).

Significance levels for all tests was \( P < 0.05 \). However, following the recommendation of Nakagawa and Cuthill (2007) we present standardized effect size estimates for all findings with \( P \geq 0.1 \), so readers can judge the biological importance of the results. Hedges' \( d \) estimates of effect size (Gurevitch and Hedges, 2006; Nakagawa and Cuthill, 2007) were calculated from the \( t \) values of our linear models using the equations provided by Nakagawa and Cuthill (2007). In order to improve the graphical representation of our results, we assigned the sign of significant main effects size estimates to represent the direction of the response of manipulated versus control microcosms (i.e. positive effect sizes indicate increases in the response variable, while negative effect sizes indicate the opposite). Further, we assigned the signs of 2-way and 3-way interaction effect size estimates to represent the classification of the interactions according to the framework proposed by Jackson et al. (2016). Thus, in our graphs positive interaction effect sizes represent synergistic interactions (i.e. the combined effect of the stressors is greater than the sum of their individual effects), whereas negative effect sizes represent either antagonistic (i.e. the combined effect of the stressors is less than the sum of their individual effects) or reversal interactions (i.e. the combined effect of the stressors is in the opposite direction than the sum of the individual effects).

We used distance-based redundancy analysis (db-RDA) to evaluate the effect of the stressor treatments on multivariate taxa composition of benthic invertebrates collected in gravel and leaf packs. Db-RDA is based on redundancy analysis (RDA), a common form of direct gradient ordination that allows to test the association between multivariate data and individual terms in a factorial experimental design. Db-RDA is specially suited for community data because it runs the RDA from a matrix of dissimilarities that can be built from ecologically relevant indices of species composition association (Legendre and Anderson, 1999; McArdle and Anderson, 2001). In our case, we used the Bray-Curtis dissimilarity index and log-transformed invertebrate abundance data prior analysis. The significance of each treatment and interaction was evaluated with a Monte Carlo permutation test of the full linear model with 999 randomizations. Water quality measures recorded over
the two weeks of the experiment were analyzed with linear mixed effects models (LME). The week of the measurement and the different stressor treatments were treated as fixed effects, while each individual microcosm was treated as a random effect. When necessary, square-root or fourth-root transformations were applied to improve normality of positively skewed distributions of count variables, and log-transformations were used to improve normality in other types of variables (Quinn and Keough, 2002). All analyses were performed in R v. 3.3.0 (R Core Team, 2016), using packages lme4 (Bates et al., 2015), car (Fox and Weisberg, 2011), and vegan (Oksanen et al., 2016).

2.3 Results

2.3.1 Water quality parameters

In agreement with our expectations, water samples collected three days after treatment application showed that nutrient additions augmented average nitrate (NO$_3$-N: 90.83 ± 25.7 µg L$^{-1}$, mean nutrient treatment; ANOVA nutrient effect: $F_{1,15} = 5.89, P = 0.029$) and phosphate (PO$_4$-P: 3.1 ± 0.7 µg L$^{-1}$, mean nutrient treatment ± SE) concentrations. However, the change in phosphate was not statistically significant at the time of collection (ANOVA nutrient effect: $F_{1,15} = 5.89, P = 0.677$). Additionally, we observed an unexpected negative effect of the insecticide application on nitrate concentrations (ANOVA insecticide effect: $F_{1,15} = 73.44, P < 0.0001$). The sediment treatment caused a six-fold increase in total suspended solids concentration that was still detectable 3 days after the application (ANOVA, $F_{1,24} = 8.11, P = 0.01$) but was negligible by the end of the experiment (ANOVA, $F_{1,24} = 0.0003, P = 0.98$). A technical failure in the chlorpyrifos determination resulted in recoveries below 1% for the chemical analysis. Consequently, we cannot report actual concentration values, but we note that despite low recoveries, chlorpyrifos was detected in all microcosms treated with the insecticide, except for those where sediment was simultaneously applied (sediment x insecticide treatment).

Temperature, pH, conductivity, and dissolved oxygen changed over the course
of the experiment, but only conductivity and dissolved oxygen were significantly affected by the treatments (Appendix Table A.1). Conductivity was higher in sediment (LME: sediment effect, $F_{1,24} = 245.5$, $P < 0.001$) and nutrient microcosms (LME: nutrient effect, $F_{1,24} = 5.3$, $P = 0.031$); and we observed a significant interaction between chlorpyrifos and nutrients for dissolved oxygen (LME: insecticide x nutrients effect, $F_{1,24} = 10.98$, $P = 0.003$). This interaction was classified as antagonistic; while both stressors tended to increase dissolved oxygen independently, their combination had no effect, resulting in average oxygen concentrations that were 1.2% lower than expected if the effect of the stressors was additive.

### 2.3.2 Invertebrate community characteristics

Invertebrate communities in control microcosms were diverse, averaging 26.6 ($\pm$11.2, SE) taxa and 126 ($\pm$ 19.5, SE) individuals per microcosm by the end of the experiment. On average, 84% of the individuals were collected in gravel (0.04 individuals per cm$^3$ of gravel, mean, $n = 4$), with the remaining 16% collected in leaf packs (15 individuals per gram of leaf litter, mean, $n = 4$) in control microcosms. In leaf packs of the control treatments, the Chironomidae subfamilies Orthocladiinae and Chironominae were the most abundant taxa, comprising 44% and 21% of the leaf invertebrate abundance, respectively. Ecclisomyia and Capnia were also an important component of the leaf pack community with 13% and 5% of the abundance, respectively. The most abundant taxa in the gravel of the control treatments were Chironominae (36%), Ecclisomyia (13%), Heterlimnius (11%), and Orthocladiinae (10%).

### 2.3.3 Effects on gravel invertebrates

Boxplots for all the biological variables evaluated in this study are presented in Appendix Figures A.2-A.4. We detected strong negative effects of sedimentation on the abundance and diversity of gravel invertebrate communities (Table 2.1, Figure 2.2). Microcosms treated with sediment had on average 26% fewer individuals and 17% less taxa than microcosms without sediment. According to our analyses these
significant effects were mostly due to negative impacts on sensitive EPT taxa (46% reduction of EPT abundance, 21% reduction of EPT richness), and large individuals (42% reduction in abundance). Further, we detected a significant antagonistic interaction between nutrients and insecticide affecting gravel invertebrate richness. Nutrient x insecticide microcosms had approximately 1.6 times more taxa than expected if the cumulative effect of two stressors was additive (Figure 2.3a). We also detected a significant interaction between sedimentation and insecticide for the abundance of small-sized invertebrates. This interaction was classified as a reversal; while the two stressors independently had weak positive effects on numbers of small-sized individuals, their cumulative effects on this metric were negative. In fact, the abundance of small individuals in sediment x insecticide tanks was approximately 2.4 times lower than expected if the two stressors were additive (Figure 2.3b).

According to the db-RDA, the treatments together explained 29% of the total variation on community composition of gravel invertebrates (Figure 2.4). Not surprisingly, sedimentation explained 8% of the variation, and was the treatment with the most significant effects on composition of gravel invertebrates (db-RDA, sediment effect: $F_{1,22} = 2.0, P = 0.007$); causing substantial reductions in the abundance of Orthocladiinae (65% reduction), Ecclisomyia (55% reduction), Paraleptophlebia (48 reduction), and Serratella (42% reduction). Nutrient additions also affected the composition of gravel invertebrates, explaining 6% of the variation in composition, but the effect was only marginally significant (db-RDA, nutrient effect: $F_{1,22} = 1.5, P = 0.071$). The nutrient treatment was negatively associated with the abundance of Ecclisomyia (48% reduction). Insecticide contamination explained 4% of the variation in taxa composition but was not deemed significant according to the Monte Carlo permutation test.

### 2.3.4 Effects on leaf pack invertebrates

We observed less severe effects of the stressors on invertebrate communities collected in leaf packs (Table 2.1, Figure 2.5). According to our linear models, the abundance of small-sized invertebrates was the only variable showing significant
effects of the stressors, with a significant sediment x insecticide interaction term. This interaction was classified as antagonistic, because the combined effects of the two stressors were 3 times less negative than predicted by additivity (Figure 2.3c). In fact, when applied as single stressors, the sediment and the insecticide treatments caused 48% and 70% reductions in the number of small individuals collected in leaf packs, respectively.

The distance-based redundancy analysis (db-RDA) detected significant interactive impacts of the treatments on the composition of leaf pack invertebrates (Figure 2.6). The treatments together explained 27% of the total variation in community composition in leaf packs, with the sediment x insecticide interaction explaining 7.3% of the total variation (db-RDA, sediment x insecticide effect: $F_{1,22} = 1.6, P = 0.027$), and the sediment x nutrient x insecticide interaction explaining 7.2% of the variation in the composition (db-RDA, nutrient x sediment x insecticide effect: $F_{1,22} = 1.6, P = 0.04$). A closer examination of Chironominae, an abundant subfamily strongly associated with the sediment x insecticide interaction term, suggested an antagonistic interaction. Sediment x insecticide tanks had on average 6.3 times higher abundance of Chironominae than predicted by additivity (Figure 2.3d). Similarly, the subfamily Orthocladiinae, which was strongly associated with the nutrient x sediment x insecticide term, showed a significant antagonistic interaction among the three stressors, as tanks with the three combined stressors had approximately the same abundance of Orthocladiinae as control tanks (Figure 2.3e).

### 2.3.5 Effects on ecosystem processes

Chlorpyrifos had strong negative effects on leaf decomposition, causing an average 21% reduction in the amount of mass lost from the leaf packs by the end of the experiment (Figure 2.7, Table 2.1). Sediment additions also depressed leaf decomposition in the microcosms, reducing mass loss by 12%. In addition, the sediment treatment caused strong negative effects on periphyton; there was 61% less periphyton biomass in microcosms treated with sediment. Contrary to our expectations, we did not detect significant effects of nutrient enrichment on leaf
decomposition or periphyton biomass in our experiment.

2.4 Discussion

Our results support the hypothesis that low concentrations of chlorpyrifos have the potential to alter freshwater ecosystem processes and interact with environmentally relevant levels of sedimentation and nutrient enrichment. Our observations are consistent with a growing body of research highlighting the importance of considering multiple-stressor interactions, and indirect effects on ecosystem processes, when evaluating the impacts of organic toxicants on freshwater ecosystems (Alexander et al., 2016; Gessner and Tlii, 2016; Schäfer et al., 2016).

2.4.1 Single stressor effects

Because the N:P ratio of Spring Creek (the stream we used as source of water, algae, and invertebrates) suggested P-limitation of the system, we predicted in our first hypothesis that a moderate pulse of phosphorus and nitrogen would have significant positive effects on functional and structural variables (Woodward et al., 2012b; Rosemond et al., 2015). Contrary to our expectations, our nutrient treatment was not sufficient to cause a response that would be detectable by the end of the experiment. However, some of our results suggest the enrichment may have had a short-term effect on the experimental systems. For example, despite the nutrient addition, there was no evidence of higher concentration of inorganic phosphorus in nutrient tanks three days after the application, which might indicate that phosphorus was quickly taken up. Consistent with this observation, nutrient enrichment tanks showed a 16% increase in periphyton biomass, but the main effect was not significant due to large variation in periphyton response in enriched tanks. These observations may suggest that the phosphorous-limited periphyton communities quickly assimilated the extra phosphorous but the subsequent increase in biomass was rapidly transferred to consumers. Modest nutrient pulses have been previously documented to have little effects on the P-limited stream communities of coastal British Columbia (Mallory and Richardson, 2005), suggesting that these
Communities tend to become P-limited again after nutrient pulses. Another factor that may have contributed to the mild response of the periphyton community is the light limitation of our study systems. Previous investigations have demonstrated that periphyton communities in headwater streams of the Malcolm Knapp Research Forest (including Spring Creek) are strongly light-limited (Kiffney et al., 2003, 2004). Even though we did not directly measure light levels in our microcosms, the average Chlorophyll-α values we observed in control microcosms of our study (0.14 ± 0.1 µg cm⁻², average ± SD) closely matched values reported in the field for undisturbed headwater streams of the Malcolm Knapp Research Forest in the fall season (0.1 ± 0.1 µg cm⁻²), suggesting similar levels of light-limitation (Kiffney et al., 2003). Thus, even if the response of the periphyton was limited by light availability in our systems, such a pattern likely recreates natural conditions of the communities under study.

Consistent with our second hypothesis, sedimentation was the most influential of the three stressors evaluated in this study; it had strong negative effects on most gravel invertebrate community metrics and affected all ecosystem processes measured in the microcosms. These observations agree with an extensive body of literature reporting negative effects of sedimentation on experimental and natural stream ecosystems (Wood and Armitage, 1997; Matthaei et al., 2006; Piggott et al., 2015b; Louhi et al., 2017). Furthermore, our results indicate that sediment additions had particularly strong effects on the pollution-sensitive EPT taxa and large-sized individuals in our experimental invertebrate communities, consistent with patterns reported in previous manipulative studies (Matthaei et al., 2006; Piggott et al., 2015b). Increased inputs of fine sediment on stream ecosystems affect sensitive invertebrates directly by reducing habitat availability, coating gills and respiratory surfaces, and impairing food quality and quantity (Wood and Armitage, 1997; Allan, 2004). On the other hand, sedimentation reduces leaf breakdown rates by altering microbial colonization and consumption by detritivorous invertebrates (Lecerf and Richardson, 2010), and limits periphyton growth by reducing light availability, while impairing substrate for recruitment (Wood and Armitage, 1997; Allan, 2004).

Contrary to our third prediction, the chlorpyrifos exposure evaluated in this
study was not sufficient to cause significant lethal effects on the aquatic invertebrate community independently. The only invertebrate metric that showed significant reductions due to the insecticide was the number of small-sized individuals in leaf packs, but this effect was only observed in absence of nutrient enrichment and sedimentation. Interestingly, even though the insecticide pulse did not cause significant invertebrate mortality in the microcosms, it still altered ecosystem function by lowering leaf decomposition rates, supporting our fourth prediction. Because chlorpyrifos is not reported to affect microbial communities at environmentally relevant concentrations (Giesy and Solomon, 2014), we attribute this result to the inhibition of invertebrate-mediated leaf decomposition. Our observations are consistent with this hypothesis, as the insecticide tended to reduce the biomass of leaf pack invertebrates in our microcosms. This reduction suggests the possibility of feeding inhibition of invertebrate shredders, a sublethal effect that has been reported in the past for other insecticides (Kreutzweiser et al., 2008, 2009; Pestana et al. 2009). This finding generally agrees with previous studies suggesting indirect negative effects of chlorpyrifos on leaf decomposition in experimental freshwater ecosystems. For example, Brock et al. (1992b) and Van den Brink et al. (1996) reported in different mesocosm experiments that chlorpyrifos substantially reduced the number of arthropod detritivores in outdoor experimental ditches. Furthermore, Cuppen et al. (1995) detected reduced decomposition rates due to detritivore mortality in similar freshwater mesocosms. More generally, these results contribute to a growing body of literature reporting indirect impacts of insecticide contamination on leaf breakdown in field and mesocosm studies (Lauridsen et al., 2006; Kreutzweiser et al., 2008; Pestana et al., 2009; Schäfer et al., 2012; Brosed et al., 2016).

On the other hand, our fifth hypothesis predicting positive effects of chlorpyrifos on primary production was not supported by our observations. This result contrasts with a number of studies reporting that chlorpyrifos increases periphyton and phytoplankton biomass through top-down effects cascading from planktonic or benthic invertebrate grazers (Ward et al., 1995; Van den Brink et al., 2009; Williams et al., 2014). However, we do not find it entirely surprising, as periphyton communities in our microcosms seemed to be nutrient and light-limited (see above).
2.4.2 Interactions between chlorpyrifos and other agricultural stressors

Even though we could not obtain a reliable estimation of chlorpyrifos concentrations in the water column of our microcosms, we observed that 36 h after the application the chemical was detected in all spiked microcosms except for those simultaneously treated with sediment. This qualitative observation agrees with an extensive body of literature documenting quick sorption of the chemical into aqueous sediment and suggests accumulation and longer persistence of the pesticide in the sediment (Brock et al., 1992b; Giesy et al., 1999; Pablo et al., 2008; Gebremariam, 2011). Our hypothesis 6 predicted that this accumulation would enhance the negative effects of the pesticide by increasing the contact exposure of invertebrates to the toxicant. We found support for this prediction in the form of a significant reversal interaction for the abundance of small gravel invertebrates. Reversal interactions have been also defined as “mitigating synergisms” (Piggott et al., 2015c) and represent one of the most extreme and interesting cases of non-additive effects; when the observed cumulative effects of two stressors are in the opposite direction than the predicted additive effects (Piggott et al., 2015c; Jackson et al., 2016). In our study, such interaction suggests that even though the effects of the insecticide alone were not significant, the presence of fine sediment in the substrate increased the exposure enough to affect small-sized invertebrates negatively. This result suggests that risk assessments assuming that invertebrates are not susceptible to chlorpyrifos when it is adsorbed to sediment could potentially underestimate its negative effects on sensitive components of the invertebrate assemblage, and highlights the importance of measuring toxicity of sediment-bound pesticides on aquatic organisms, a risk factor that is often overlooked (Warren et al., 2003).

Furthermore, our results are in keeping with previous reports of size-dependent sensitivity of aquatic invertebrates to pollutants (Kiffney and Clements, 1996b,a), since the reversal interaction was only observed on the smaller size class of our experimental invertebrate communities. Smaller invertebrates are more sensitive to pollutants due to larger surface area to volume ratios, higher mass-specific metabolism
that potentially accelerates uptake of the toxicant, and frequent molting during early larval stages (Kiffney and Clements, 1996a; Townsend and Thompson, 2007).

Interestingly, our findings indicate that for leaf pack invertebrates the presence of fine sediment in the substrate had the opposite effect, mitigating the negative impacts of the insecticide on the abundance of small-sized invertebrates and the richness of sensitive taxa. This observation may indicate that with added fine sediment in the substrate, there was less chlorpyrifos exposure in the leaf pack microhabitat, which mitigated the negative effects of the toxicant on the leaf community. Previous empirical research on chlorpyrifos has also reported that physical characteristics of the ecosystem, such as the presence or absence of dominant macrophytes can substantially modify the exposure of organisms to chlorpyrifos thus modifying the nature and magnitude of the impacts of the insecticide (Brock et al., 1992b).

In agreement with hypothesis 7, we detected a significant antagonistic interaction between nutrient enrichment and chlorpyrifos contamination for gravel invertebrate richness. Even though both stressors tended to reduce gravel invertebrate richness, their combination did not produce additive taxa losses in the system. Instead, both stressors tended to eliminate the same sensitive taxa (e.g., Ameletus, Cinygmula, Baetidae), thus producing less losses than predicted by additivity. This type of antagonistic interaction suggests that the invertebrate community under study presents positive species co-tolerance to eutrophication and chlorpyrifos (Vinebrooke et al., 2004). These observations contrast with a previous investigation reporting additive effects of chlorpyrifos and eutrophication in indoor microcosms modelled after Dutch drainage systems (Traas et al., 2004). However, a mesocosm study evaluating interactions between a tertiary mixture of insecticides (including chlorpyrifos) and nutrient enrichment, also reported mitigating interactions at moderate levels of nutrient enrichment and sublethal levels of the insecticide mixture (Alexander et al., 2013). Furthermore, they detected that such mitigating interactions turned into synergistic after a threshold of nutrient enrichment that was species-specific (Alexander et al., 2013). These contrasting lines of evidence further highlight that the outcome of multiple stressor interactions is strongly context-dependent (Clements et al., 2016), and deserve further investigation to unravel the mechanisms and determine thresholds where eutrophication levels start interact-
ing synergistically with insecticide concentrations that are deemed safe for aquatic ecosystems.

2.4.3 Implications

Community-level microcosm and mesocosm experiments can incorporate enough ecological complexity to test indirect effects of the stressors, while providing relatively controlled conditions to isolate relevant variables, as well as sufficient replication for rigorous hypothesis testing (Culp et al., 2000; Clements, 2004). However, the extrapolation of our experimental results to field scenarios should be done with care. Several simplifications imposed by the logistical constraints of our experiment, such as the recirculating flow, the hydraulic conditions in the tanks, the relatively short duration of the test, and the closed community population dynamics (without immigration or emigration), may result in overestimation or underestimation of the negative impacts of the stressor pulses. For instance, the duration of the insecticide exposure caused by a single pulse of chlorpyrifos, is likely to be longer in recirculating microcosms than in natural environments, where the insecticide is quickly transported downstream. Hence, the effects of the pesticide on a single habitat patch may be overestimated. However, with these caveats in mind, our study still offers some interesting insights into the single and interactive impacts of chlorpyrifos at the microhabitat scale, revealing indirect effects and complex multiple stressor interactions that deserve further investigation. Our results support a body of evidence showing that multiple stressor interactions should be explicitly considered when conducting environmental risk assessments for insecticides, as even at low insecticide concentrations, synergistic interactions with other common agricultural stressors may result in unexpected negative effects for aquatic invertebrate communities (Alexander and Culp, 2008; Alexander et al. 2016). Furthermore, the differential effects of the insecticide on habitat patches, highlight the importance of habitat structure in modulating the impacts of organic toxicants on non-target species, keeping with a growing body of literature suggesting that in-stream habitat conditions must be taken into account when conducting environmental risk assessments for pesticides (Rasmussen et al., 2011, 2012; Schäfer
et al., 2016). Additionally, our detailed study at the scale of microhabitats allowed us to document chlorpyrifos-induced effects on invertebrate communities inhabiting leaf packs, along with lower leaf decomposition rates. These results are in keeping with field studies correlating leaf breakdown inhibition with pesticide exposures (Schäfer et al., 2007, 2012; Rasmussen et al., 2012; Brosed et al., 2016), and offer a mechanistic explanation for such impacts, underscoring the indirect effects of insecticides through invertebrate decomposers at sublethal concentrations. Moreover, our results strongly support the notion that leaf decomposition is a useful early indicator of ecological impairment in stream ecosystems and should complement the suite of structural measurements already employed in biomonitoring (Gessner and Chauvet, 2002; Pestana et al., 2009; Schäfer et al., 2012; Brosed et al., 2016).
Table 2.1: ANOVA summary of linear fixed effects models evaluating impacts of stressor treatments on ecosystem functioning and invertebrate variables recorded on day 15 of the experiment. Significant effects are indicated in bold ($P < 0.05$).

<table>
<thead>
<tr>
<th>Response variables</th>
<th>df</th>
<th>N</th>
<th>S</th>
<th>I</th>
<th>N*S</th>
<th>N*I</th>
<th>S*I</th>
<th>N<em>S</em>I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ecosystem responses</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Leaf decomposition</td>
<td>1, 24</td>
<td>2.881</td>
<td>0.103</td>
<td>4.592</td>
<td><strong>0.042</strong></td>
<td>8.864</td>
<td><strong>0.007</strong></td>
<td>0.648</td>
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<tr>
<td>Periphyton biomass</td>
<td>1, 24</td>
<td>0.207</td>
<td>0.653</td>
<td>8.215</td>
<td><strong>0.009</strong></td>
<td>2.516</td>
<td>0.126</td>
<td>0.665</td>
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<td><strong>Gravel invertebrates</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abundance</td>
<td>1, 22</td>
<td>2.999</td>
<td>0.097</td>
<td>4.645</td>
<td><strong>0.042</strong></td>
<td>0.094</td>
<td>0.762</td>
<td>0.001</td>
</tr>
<tr>
<td>Biomass</td>
<td>1, 22</td>
<td>0.018</td>
<td>0.893</td>
<td>1.298</td>
<td>0.267</td>
<td>0.167</td>
<td>0.687</td>
<td>0.105</td>
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<td>1, 22</td>
<td>3.935</td>
<td>0.060</td>
<td>6.504</td>
<td><strong>0.018</strong></td>
<td>1.002</td>
<td>0.328</td>
<td>0.437</td>
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<tr>
<td>EPT abundance</td>
<td>1, 22</td>
<td>2.999</td>
<td>0.097</td>
<td>4.645</td>
<td><strong>0.042</strong></td>
<td>0.094</td>
<td>0.762</td>
<td>0.001</td>
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<tr>
<td>EPT biomass</td>
<td>1, 22</td>
<td>0.000</td>
<td>0.986</td>
<td>0.565</td>
<td>0.460</td>
<td>1.134</td>
<td>0.298</td>
<td>0.064</td>
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<tr>
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<td>0.176</td>
<td>4.399</td>
<td><strong>0.048</strong></td>
<td>2.225</td>
<td>0.150</td>
<td>0.009</td>
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<td>Average body size</td>
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<td>2.726</td>
<td>0.113</td>
<td>1.876</td>
<td>0.185</td>
<td>0.191</td>
<td>0.667</td>
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<td>Abundance small size</td>
<td>1, 22</td>
<td>3.766</td>
<td>0.065</td>
<td>3.196</td>
<td>0.088</td>
<td>0.203</td>
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<td>Abundance large size</td>
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<td>0.001</td>
<td>0.980</td>
<td>4.798</td>
<td><strong>0.039</strong></td>
<td>0.132</td>
<td>0.720</td>
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<td><strong>Leaf invertebrates</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abundance</td>
<td>1, 22</td>
<td>0.050</td>
<td>0.825</td>
<td>0.002</td>
<td>0.968</td>
<td>0.940</td>
<td>0.343</td>
<td>0.368</td>
</tr>
<tr>
<td>Biomass</td>
<td>1, 22</td>
<td>0.005</td>
<td>0.942</td>
<td>0.117</td>
<td>0.736</td>
<td>2.993</td>
<td>0.098</td>
<td>0.203</td>
</tr>
<tr>
<td>Richness</td>
<td>1, 22</td>
<td>0.174</td>
<td>0.681</td>
<td>0.113</td>
<td>0.740</td>
<td>0.763</td>
<td>0.392</td>
<td>0.157</td>
</tr>
<tr>
<td>EPT abundance</td>
<td>1, 22</td>
<td>0.889</td>
<td>0.356</td>
<td>0.013</td>
<td>0.910</td>
<td>0.978</td>
<td>0.333</td>
<td>0.887</td>
</tr>
<tr>
<td>EPT biomass</td>
<td>1, 22</td>
<td>2.535</td>
<td>0.126</td>
<td>0.626</td>
<td>0.437</td>
<td>2.050</td>
<td>0.166</td>
<td>0.165</td>
</tr>
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<td>EPT richness</td>
<td>1, 22</td>
<td>1.202</td>
<td>0.285</td>
<td>0.079</td>
<td>0.781</td>
<td>0.934</td>
<td>0.344</td>
<td>1.455</td>
</tr>
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<td>Average body size</td>
<td>1, 22</td>
<td>0.876</td>
<td>0.360</td>
<td>0.016</td>
<td>0.900</td>
<td>1.546</td>
<td>0.227</td>
<td>0.000</td>
</tr>
<tr>
<td>Abundance small size</td>
<td>1, 22</td>
<td>0.299</td>
<td>0.590</td>
<td>0.004</td>
<td>0.948</td>
<td>0.913</td>
<td>0.350</td>
<td>0.913</td>
</tr>
<tr>
<td>Abundance large size</td>
<td>1, 22</td>
<td>1.123</td>
<td>0.301</td>
<td>2.201</td>
<td>0.152</td>
<td>0.719</td>
<td>0.406</td>
<td>0.719</td>
</tr>
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</table>
Figure 2.1: Photo of the experimental set-up consisting in 32 laboratory microcosms located at the University of British Columbia, Vancouver, Canada (a). Detail of the microcosms (b).
Figure 2.2: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant main and interactive effects of the stressors on gravel invertebrate communities. Letters in the y-axis represent main effects (N, nutrients; S, sediment; I, insecticide) and interaction terms (N*S, nutrient x sediment; N*I, nutrient x insecticide; S*I, sediment x insecticide; N*S*I; nutrient x sediment x insecticide). For main effects, significant (i.e. not overlapping zero) positive values indicate increases in the response variable whereas negative values denote the opposite. For interactions, confidence intervals overlapping zero indicate additive interactions, positive values indicate synergies, and negative values indicate antagonistic or reversal interactions (reversals are marked with an R). Symbols are used to represent significance of the effects according to the ANOVAs: *$P < 0.05$, ·$P < 0.1$. 

*34
Figure 2.3: Bar plots illustrating significant interactive effects of the stressors on gravel (a-b) and leaf (c-e) invertebrate metrics, according to the ANOVAs ($P = 0.05$). All shown interactions were classified as antagonistic, except for panel (b) which was classified as a reversal. Letter notation for the treatments is consistent with Figure 2.2. Bars represent the mean of four replicates ($\pm$ SE).
Figure 2.4: Distance-based redundancy analysis on log-transformed abundance data of invertebrates collected in gravel. Only the most abundant taxa are labeled. Letter notation for the treatments is consistent with Figure 2.2. Solid arrows indicate significant main effects ($P < 0.05$) and dashed arrows indicate marginally significant effects ($P = 0.059$), according to a permutation test of the full model with 999 randomizations. The treatments together explained 29% of the total variation on community composition of gravel. Axes 1 and 2 represent 10 and 5% of the total variation, respectively.
Figure 2.5: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant main and interactive effects of the stressors on invertebrate communities collected in leaf packs. Letter notation for the treatments and interpretation of effects is consistent with Figure 2.2. Symbols are used to represent significance of the effects according to the ANOVAs: *$P < 0.05$, †$P < 0.1$. 
Figure 2.6: Distance-based redundancy analysis on log-transformed abundance data of invertebrates collected in leaf packs. Only the most abundant taxa are labeled. Letter notation for the treatments is consistent with Figure 2.2. Solid arrows indicate significant main effects ($P < 0.05$) according to a permutation test of the full model with 999 randomizations. The treatments together explained 27% of the total variation on community composition of leaf packs. Axes 1 and 2 represent 7 and 6% of the total variation, respectively.
Figure 2.7: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant main and interactive effects of the stressors on ecosystem processes. Letter notation for the treatments and interpretation of effects is consistent with Figure 2.2. Symbols are used to represent significance of the effects according to the ANOVAs: $**P < 0.01$, $*P < 0.05$. 
Chapter 3

Nutrients and sediment modify the impacts of a neonicotinoid insecticide on experimental pond ecosystems

3.1 Introduction

Freshwater ecosystems are amongst the most threatened ecosystems in the world (Dudgeon et al., 2006; Vorösmarty et al., 2010; Strayer and Dudgeon, 2010). Threats to freshwaters have been extensively documented, with habitat degradation and loss, nutrient enrichment, flow modification, overexploitation, introduction of non-native species, and chemical pollution figuring as some of the stressors driving the declining trends (Dyke, 2008; Vorösmarty et al., 2010; Schäfer et al., 2016). With the increasing recognition that these stressors rarely operate in isolation, the last decade has seen growing interest in the experimental assessment of their cumulative effects. This experimental approach has improved our mechanistic understanding of the impacts of stressors like nutrient enrichment, sedimenta-
tion, temperature, acidification, and water abstraction (e.g., Matthaei et al., 2010; Piggott et al., 2015b; Alexander et al., 2016). Most importantly, this approach has demonstrated that the combined action of these stressors frequently results in impacts that cannot be predicted from the addition of the stressors’ individual effects, highlighting the importance of this type of experimental work to understand and predict complex multiple-stressor interactions (Wagenhoff et al., 2012; Shurin et al., 2012; Piggott et al., 2015b; Jackson et al., 2016).

Despite these advances, our understanding about cumulative effects of some important freshwater ecosystem stressors is still relatively limited. Neonicotinoid insecticides are a clear example of this situation. Neonicotinoid insecticides were introduced as systemic pest controls in the early 1990s, but their large-scale application started around 2004 and has rapidly increased to the point that they are currently the most widely used insecticide family in the world (Main et al., 2014; Simon-Delso et al., 2015). They have been the object of great controversy in the last few years, as they have been associated with the colony collapse disorder causing alarming honey bee declines around the world, and have been attributed to toxic effects on mammals (Köhler and Triebeskorn, 2013). These controversial findings have raised interest in these insecticides, and a number of studies have addressed their potential negative effects on non-target organisms, including some freshwater species (e.g., Kreutzweiser et al., 2008; Langer-Jaesrich et al., 2010; Churchel et al., 2011; Böttger et al., 2013). However, to date there is relatively little empirical information about interactions between neonicotinoids and other agricultural stressors in freshwater ecosystems (but see Alexander et al., 2013, 2016).

Neonicotinoid insecticides are frequently detected in puddles, irrigation channels, streams, rivers, and wetlands in agricultural lands of different countries (Main et al., 2014; Morrissey et al., 2015; Sánchez-Bayo et al., 2016). In these water bodies, freshwater communities are simultaneously exposed to other agricultural stressors that may affect their sensitivity to the neonicotinoid insecticides. For example, some agricultural practices result in increased inputs of fine sediment into water bodies (Allan, 2004). Elevated inputs of fine sediment increase water turbidity reducing light penetration and primary production, impair substrata for invertebrate habitat and periphyton growth, and smother respiratory organs of sen-
sitive invertebrate taxa (Knowlton and Jones, 1995; Wood and Armitage, 1997; Matthaei et al., 2006). On the other hand, the application of fertilizers in agricultural landscapes often causes nutrient enrichment of freshwater ecosystems (Allan, 2004). At moderate levels this enrichment has positive bottom-up effects in freshwater food webs, but at high levels they might lead to extensive algae growth and consequent decreases in dissolved oxygen that ultimately result in loss of sensitive species (Allan, 2004; Wagenhoff et al., 2012; García et al., 2017). There are multiple mechanisms by which neonicotinoids may interact non-additively with sedimentation and nutrient enrichment. For example, imidacloprid, the most popular insecticide in this family, is relatively soluble in water (610 mg L\(^{-1}\) in 20°C H\(_2\)O; \(\log K_{ow} = 0.57\)), but it tends to persist longer in sediment than in water (water half-life under light conditions: 4 h, sediment half-life under anaerobic conditions: 27 days; CCME, 2007). Thus, imidacloprid may interact synergistically with sedimentation in agricultural landscapes, if its longer persistence in sediment increases contact exposure of benthic organisms to the insecticide. Furthermore, a recent experiment reported that high levels of nutrient enrichment were able to mask the negative effects of imidacloprid on benthic invertebrate communities (Alexander et al., 2016).

In addition to potential multiple-stressor interactions, some experiments have suggested the possibility of indirect impacts of sublethal levels of imidacloprid contamination on important freshwater ecosystem processes. Using single-species microcosms and outdoor stream mesocosms, respectively, Kreutzweiser et al. (2008) and Pestana et al. (2009) demonstrated that imidacloprid contamination reduced leaf breakdown rates through feeding inhibition of detritivorous invertebrates. Moreover, Alexander et al. (2016) observed in their stream mesocosm experiment that imidacloprid contamination enhanced the positive indirect impacts of predation on periphyton biomass. These findings underscore that even sublethal concentrations of this common insecticide may alter primary productivity and organic matter dynamics in freshwater ecosystems, and highlight the need for more empirical studies explicitly assessing the effects of the insecticide on integrative measurements of ecosystem functioning, such as ecosystem metabolism.

Here, we present the results of an ecosystem-scale, mesocosm experiment de-
signed to evaluate individual and combined effects of imidacloprid, sedimentation, and nutrient enrichment on structure and function of pond ecosystems. To our knowledge, this is the first time these three stressors have been manipulated simultaneously, thus we were particularly interested in testing whether their combined effects could be predicted from the addition of their individual effects. Additionally, we evaluated indirect impacts of the insecticide on ecosystem metabolism and organic matter decomposition. We predicted that at the stressor levels we tested in our study, we would observe: 1) positive individual effects of nutrients on leaf decomposition, primary production, and invertebrate biomass; 2) negative individual effects of sedimentation on most community metrics and ecosystem processes; 3) negative effects of imidacloprid on benthic invertebrate density; 4) no effects of imidacloprid on zooplankton density; 5) indirect positive effects of imidacloprid on net ecosystem productivity through the inhibition of grazers; 6) indirect negative effects of imidacloprid on leaf decomposition through the inhibition of shredders; 7) antagonistic interactions between imidacloprid and nutrient enrichment mitigating negative effects on invertebrate communities; and 8) synergistic interactions between imidacloprid and sedimentation exacerbating negative impacts on invertebrate communities.

3.2 Methods

3.2.1 Experimental design

We conducted a 36-day factorial manipulation of fine sediment, nutrients, and imidacloprid concentration in 32 outdoor freshwater mesocosms (1136 L plastic tanks, 0.6 m deep, 1.4 m in diameter; Rubbermaid®, Atlanta, GA, USA), located at the University of British Columbia’s experimental pond facility, Vancouver, Canada (49° 14’ 52.1” N, 123° 13’ 55.9” W; Figure 3.1). Four months before the onset of the experiment, tanks were filled with 616 L of municipal water and left to dechlorinate by degassing for three weeks. Then they were stocked with substrate and organic matter to provide suitable habitat for invertebrates, this included: a 3 cm layer of medium sand (< 5 mm grain size) and washed mixed gravels (0.5-2
cm grain size range), 25 g of rabbit pellets, and 50 g of air-dried red alder (*Alnus rubra*) leaves. Three months prior to treatment application, each mesocosm was inoculated with 10 L of unfiltered lake water and a 1 L aliquot of concentrated live plankton (collected using a 64 µm mesh conical tow net) from local lakes, to provide colonists of zooplankton and phytoplankton. Similarly, 1 L of benthic sediments from local lakes were applied to each mesocosm to provide colonists of benthic invertebrates. All collected sediment, water, and zooplankton was mixed thoroughly before addition to ensure mesocosms were receiving similar planktonic and benthic communities. Additionally, three weeks after inoculation, 40 L of water were exchanged between each tank and each of the six tanks closest to it, in order to homogenize planktonic communities. All tanks were left uncovered throughout the experiment to allow natural colonization and emigration of aquatic invertebrates. Water levels were maintained by natural precipitation and addition of equal volumes of dechlorinated municipal water to each tank once a week. According to handheld probe measurements conducted one day before the application of the treatments, there were no significant differences (*P > 0.05*) in dissolved oxygen (8.81 ± 0.1 mg L\(^{-1}\), mean ± SE, *n* = 32), conductivity (35.4 ± 0.42 µS cm\(^{-1}\)), and pH (7.7 ± 0.06), among tanks assigned to different stressor treatments.

### 3.2.2 Stressor treatments

Our experimental manipulation was conducted in the summer season, from June 4 to July 11 of 2015. The experimental design involved two levels of each stressor (added, ambient) in a fully crossed factorial design (2 x 2 x 2), with eight treatment combinations and four replicates per treatment. Treatments were randomly assigned to mesocosms and designed to simulate pulsed exposures, similar to those experienced by pond ecosystems in agricultural landscapes, due to fluctuation in rainfall and runoff events, seasonal application of fertilizers and pesticides, and accidental spills. Thus, fine sediment was added weekly, starting on June 4 (day 1), while nutrients and insecticide were applied only twice throughout the experiment (days 1 and 23).

We used powdered kaolin (Ward’s® Kaolin, 74 µm mesh size) for the sedi-
ment treatment. Kaolin is an inorganic clay with similar optical and physical properties to naturally-occurring suspended silts (Boubée et al., 1997), which is often used in studies of suspended solids in aquatic ecosystems (e.g., Boubée et al., 1997; Sanpera-Calbet et al., 2012). It has a pH of 7.5, an average organic carbon content of 14%, and a volume-specific mass of 2.6 g cm\(^{-3}\). We added 90 g of kaolin in each sediment-treatment tank once a week. To promote particle suspension, prior to addition we mixed the kaolin for each tank in 1 L of water from the same tank in a separate container. The water-kaolin mix was then added as evenly as possible onto the surface of the treated tank, and the water column was stirred. For stirring, we used a 40 cm-long PVC pipe (3/4” diameter), which was immersed in the water half-length and rotated clockwise three times without touching the tank walls. To control for a potential confounding effect of the stirring process, we also stirred the non-sediment treatment tanks on the days of sediment additions. Each tank had its own PVC mixer to avoid cross-contamination.

Background nutrient concentrations (NH\(_4\)-N: 44.8 ± 1.2 µg L\(^{-1}\), NO\(_3\)-N: 100.5 ± 46.3 µg L\(^{-1}\), PO\(_4\)-P: 4.9 ± 0.5 µg L\(^{-1}\)) were augmented by adding potassium phosphate (target P concentration: 50 µg L\(^{-1}\) above background concentration) and ammonium nitrate (target N concentration: 1500 µg L\(^{-1}\) above background concentration) on days 1 and 23 of the experiment. A stock solution of 1.2 g L\(^{-1}\) imidacloprid was prepared by dissolving analytical grade imidacloprid (≥100%, Pestanal®, Sigma-Aldrich) in deionized water. We applied 1.8 mL of the stock insecticide solution to each insecticide tank using a micropipette on days 1 and 23, for target imidacloprid pulses of 3.5 µg L\(^{-1}\). Our target imidacloprid pulses fall within the range of concentration values reported for neonicotinoid insecticides in surface waters of different agricultural regions in North America (Giroux, 2003; Starner and Goh, 2012; Anderson et al., 2013).

### 3.2.3 Response variables

We measured variables reflecting the effect of the treatments on habitat condition, pelagic and benthic communities, and ecosystem function. Water samples were collected 3 hours following treatment additions on day 1 and again on day 36 to de-
termine imidacloprid and nutrient concentrations (PO$_4$-P, NH$_4$-N, and NO$_3$-N) in the tanks. Nutrients were analyzed by the Analytical Chemistry Laboratory of the British Columbia Ministry of Environment, Victoria, BC, using standard methods (APHA 2005). Water samples for imidacloprid determination were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada, Saskatoon, SK, using the methods described in Main et al. (2014). Measures of dissolved oxygen concentration (DO), conductivity, and pH were recorded weekly using hand-held probes after the application of treatments. Weekly water samples (100 mL) were transported to the laboratory to measure turbidity using a digital desktop turbidity meter. Sedimentation rates were measured in the mesocosms by fixing three uncapped Falcon tubes vertically in different random points of the bottom of each tank. Falcon tubes were recovered on day 36, capped, and transported to the laboratory for drying (40°C for 5 d) and weighing.

We sampled pelagic communities weekly to estimate phytoplankton and zooplankton biomass. Phytoplankton biomass was estimated by collecting 2 L samples from the water column of each tank in opaque plastic containers. Containers were transported to the lab, where the water was filtered onto precombusted glass fiber filters. Filters were transferred to 15 mL Falcon tubes and covered with 90% acetone during 24 h in the dark (at 4°C), where they were homogenized by agitation three times during the steeping period. Once the steeping period was completed, samples were analyzed for chlorophyll-α in a Turner Designs TD-700 laboratory fluorometer according to standard fluorometric methods (Arar and Collins 1997). Zooplankton biomass was estimated from one composite sample per tank collected with a small conical tow net (26.6 cm opening diameter, 64 μm mesh). Each composite sample consisted of two random 30 cm-deep tows that were immediately fixed in 70% ethanol and transported to the laboratory. In the laboratory, samples were divided in half using a Folsom plankton splitter. One half of the sample was inspected under the dissecting microscope to remove all pieces of organic matter that were not zooplankton (exuviae, winged insects, seeds, etc.) and filtered onto a glass microfiber filter (Whatman® GF/F). Filters were dried at 60°C for 5 days, weighed, ashed at 500°C, and reweighed to estimate zooplankton ash-free dry mass (AFDM). For days 26 and 33 the remaining half of the sample was used
for zooplankton counting and identification, using a Bogorov counting chamber under a dissecting microscope. A 5 mL Hensen-Stempel pipette was used to obtain subsamples until at least 200 individuals were identified and enumerated, in order to estimate zooplankton densities. Zooplankters were identified to the lowest practical taxonomic resolution, usually genus.

We sampled benthic communities only once, at the end of the experiment (day 36). To estimate periphyton biomass we introduced three unglazed, 84 cm$^2$ ceramic tiles in each mesocosm on day 0 and collected them on day 36. Tiles were stored in the dark at -18°C until processing for chlorophyll-$a$ estimation through fluorometry following the methods described above. Benthic invertebrates were sampled by introducing one cylindrical benthic trap (15 cm diameter, 10 cm high) in each mesocosm two weeks before the application of the treatments. Each trap consisted of a circular plastic bottom with coarse-mesh plastic walls (square mesh fence, 1 cm mesh size), filled with a 3 cm layer of mixed gravels and 1 g of air-dried red alder leaves. On day 36, we extracted the traps with the help of a D-net (250 µm mesh size) to prevent the loss of invertebrates and washed their contents through a 2 mm sieve stacked on top of a 250 µm sieve. Gravel retained in the 2 mm sieve was sorted for invertebrates at the time of collection, and the material retained in the 250 µm sieve was stored in 70% ethanol and sorted later under the dissecting microscope. Invertebrates were enumerated, identified to the lowest practical taxonomic level (usually genus), and measured to determine size and dry mass from length-mass regressions (Smock, 1980; Benke et al., 1999; Johnston and Cunjak, 1999). Finally, invertebrates were classified into three size categories: small (< 2.5 mm), medium (2.5 - 5 mm), and large (> 5 mm).

3.2.4 Ecosystem function

One day before treatment application (day 0), we introduced three leaf packs of red alder leaves in coarse-mesh bags (3 g air-dry weight; 10 mm mesh) into each mesocosm to measure leaf decomposition. On day 36, leaf packs were removed from the mesocosms and stored at -18°C until processed in the laboratory to estimate remaining AFDM, according to standard methods (Hauer and Lamberti).
Posterior processing involved defrosting, rinsing, and drying at 60°C for 5 days. Remaining leaf material was then weighed, ashed at 500°C, and reweighed to calculate AFDM.

Net ecosystem production (NEP) was measured four times throughout the experiment (days 7, 22, 29, and 35) using gas-exchange methods. NEP was estimated as the difference in DO concentration between dusk and dawn, reflecting the rate of change in DO between the hours with maximum (dusk) and minimum (dawn) concentrations. DO measurements were recorded hourly for the full 24-h cycle on the first sampling date, in order to determine the hours of the day at which maximum and minimum DO concentrations were detected in the tanks. For the remaining sampling dates, tanks were sampled only at such hours when maximum and minimum values were recorded; 17:00 and 7:00, respectively.

### 3.2.5 Data analysis

We used linear models to quantify the individual and combined effects of nutrient enrichment, sedimentation, and insecticide contamination on univariate responses. Variables with one measurement per mesocosm were analyzed with fixed effects models, including the three stressor treatments and all the possible two-way and three-way combinations among them. Variables with multiple measurements per mesocosm were analyzed with linear mixed effects models (LME) that also included the individual mesocosm and the sampling date as random effects. Using LMEs for response variables with repeated measurements, allowed us to focus on effects that were significant across the duration of the experiment, while facilitating the collective interpretation of responses measured at different time intervals.

Significance levels for all our tests was $P < 0.05$ and was evaluated with ANOVA type III sum of squares. Following the recommendation of Nakagawa and Cuthill (2007) we present standardized effect size estimates for all findings with $P < 0.1$, so readers can judge the biological importance of the results. Hedges $d$ estimates of effect size (Gurevitch and Hedges, 2006) were calculated from the $t$ values of our linear models using the formulas provided by Nakagawa and Cuthill (2007).
In order to improve the graphical representation of our results, we coded significant main effects to represent the direction of the response of manipulated versus control mesocosms (i.e. positive effect sizes indicate increases in the response variable, while negative effect sizes indicate the opposite). Further, we coded 2-way and 3-way interaction effect sizes to represent the classification of the interaction according to the framework proposed by Jackson et al. (2016). Thus, positive interaction effect sizes represent synergistic interactions (i.e. the combined effect of the stressors is greater than the sum of their individual effects), whereas negative effect sizes represent either antagonistic interactions (i.e. the combined effect of the stressors is less than the sum of their individual effects) or reversal interactions (i.e. the combined effect of the stressors is in the opposite direction than the sum of the individual effects).

We used redundancy analysis (RDA) to evaluate the effect of the stressor treatments on multivariate taxa composition of zooplankton and benthic invertebrates. Zooplankton density and benthic invertebrate abundance were Hellinger-transformed to reduce the influence of rare taxa (Legendre and Gallagher, 2001). Significance of main effects and interactions was assessed with Monte Carlo permutations of the full model with 999 randomizations. We also used redundancy analysis (RDA) to evaluate if the treatments affected the distribution of biomass across the pelagic and benthic food webs in the ponds. In order to facilitate comparisons, biomass units from all compartments were converted into g C (g C m$^3$ for pelagic organisms, g C m$^2$ for benthic organisms). We assumed a carbon biomass to chlorophyll biomass ratio of 40:1 for phytoplankton and 50:1 for periphyton (Shurin et al., 2012). For zooplankton we had AFDM, so we assumed an average 10% of ash content (Waters, 1977), and an average carbon content of 48% of dry mass (Andersen and Hessen, 1991). For benthic invertebrates we assumed an average carbon content 48.3% of dry mass (Evans-White et al., 2005).

When necessary, root transformations were applied to improve normality of count variables with positively skewed distributions. Log-transformations were used to improve normality and homoscedasticity in other types of variables (Quinn and Keough, 2002). All analyses were performed in R v. 3.3.0 (R Core Team, 2016), using packages lme4 (Bates et al., 2015), car (Fox and Weisberg, 2011),
3.3 Results

3.3.1 Water quality and habitat characteristics

Water samples collected on day one, three hours after treatment application, showed that nutrient additions caused significant increases in phosphate (609% increase, PO$_4$-P: 35.1 ± 0.6 µg L$^{-1}$, mean nutrient treatment ± SE, n = 16, Table 3.1) and ammonia nitrogen concentrations (939% increase, NH$_4$-N: 465.6 ± 10.9 µg L$^{-1}$). Nutrient additions also increased mean nitrate concentrations (125% increase, NO$_3$-N: 226.4 ± 75.5 µg L$^{-1}$) but the effect was not statistically significant, probably due to the large variation in nitrate levels recorded in nutrient addition tanks. On the other hand, the insecticide treatment had a significant effect on nitrate, causing a 76% reduction in measured concentration values. Nutrient additions also tended to reduce DIN:DIP ratio (29% reduction) but again, the effect was not significant at the time of collection (DIN:DIP: 20.1 ± 2.7). Three hours after the first insecticide application, average imidacloprid concentration in the water was 1.97 ± 0.01 µg L$^{-1}$ in insecticide tanks (Table 3.2). Even though this exposure level was lower than our target concentration (3.5 µg L$^{-1}$), it was consistent across all tanks treated with insecticide. Average imidacloprid concentration on day 35 (12 days after the second insecticide pulse) was 0.071 ± 0.01 µg L$^{-1}$, indicating that despite the expected decrease in imidacloprid concentration after each pulse, there was some level of insecticide exposure in the treated tanks throughout the duration of the experiment.

According to biweekly turbidity measurements, the sediment addition caused an average six-fold increase in turbidity across the duration of the experiment (Table 3.1). The sediment treatment also resulted in significantly higher sedimentation rates; sediment tanks had on average 2.4 times more sediment accumulated in the substrate by the end of the experiment. According to weekly daytime measurements, water in nutrient-enriched mesocosms had 14% more dissolved oxygen and
8% higher pH than the rest of the mesocosms ($P < 0.05$). Insecticide applications also caused significant increases in water pH (4% increase). Conductivity was not significantly affected by any of the treatments throughout the experiment.

### 3.3.2 Pelagic community

Our weekly biomass measurements showed that the stressors had strong independent and interactive effects on the biomass of the pelagic community, however, their effects varied through time (Figure 3.2a-d). Nutrient enrichment was the most influential stressor, causing increases in zooplankton and phytoplankton biomass starting one week after the second nutrient pulse in the case of phytoplankton, and two weeks after the first nutrient pulse in the case of zooplankton. Linear mixed effects models controlling for time (Figure 3.3, Table 3.3), showed that across the duration of the experiment, nutrient enrichment tripled phytoplankton biomass (231% increase, Figure 3.3a) and doubled zooplankton biomass (102% increase), whereas sedimentation reduced the latter by 28% (Figure 3.3b). Furthermore, density and diversity recorded in the last two weeks of the experiment indicated that insecticide pulses reduced zooplankton density by 63% (Figure 3.3c), while sediment pulses had negative effects on richness (12% reduction, Figure 3.3d).

Our results indicate that the effects of the three stressors on the zooplankton community were not independent. We detected significant antagonistic interactions between sediment and insecticide, and between the three stressors affecting zooplankton richness in the ponds (Figure 3.3d). Zooplankton richness in sediment x insecticide mesocosms was 2 times higher than expected if the two stressors were independent. Similarly, richness in mesocosms receiving the three stressor treatments was on average 2.4 times higher than expected if the three stressors were additive (Figure 3.4a). The Shannon-Wiener and the Evenness diversity indices also showed significant 3-way antagonistic interactions; the three stressors combined produced Shannon-Wiener diversity 3.15 times higher and Evenness 1.5 times higher than expected if their impacts were additive (Figure 3.4b, c).

The redundancy analysis (RDA) showed that sediment and insecticide addi-
tions significantly altered the composition of zooplankton communities in the mesocosms by the end of the experiment (Figure 3.5a). Insecticide pulses were strongly correlated with lower densities of *Bosmina* and higher densities of Chydoridae and *Diaphanosoma*; while sediment additions increased *Diaphanosoma* and reduced *Daphnia* densities.

### 3.3.3 Benthic community

Benthic invertebrate samples collected at the end of the experiment showed that the sediment pulses caused a 32% reduction in invertebrate abundance (Figure 3.6a), mainly due to negative impacts on the number of small (61% reduction, Figure 3.6b) and medium-sized individuals (35% reduction, Figure 3.6c). However, despite having stronger impacts on the smaller size categories, the application of sediment did not alter the average size of the benthic invertebrate community (Table 3.3). Sediment pulses also had marginally significant (*P* = 0.07) impacts on invertebrate taxa richness (10% reduction, Figure 3.6e), but did not significantly affect Shannon-Wiener diversity or Evenness. Nutrient additions had positive effects on the benthic invertebrate community, increasing the number of invertebrate taxa in the mesocosms by 20% (Figure 3.6e). We did not detect strong independent effects of the insecticide pulses on benthic invertebrate communities. However, imidacloprid caused a marginally significant reduction of the abundance of medium-sized individuals (35% reduction, Figure 3.6c), and interacted non-additively with sedimentation and nutrient enrichment (Figure 3.6d). We observed a significant sediment x insecticide reversal interaction affecting the abundance of large individuals; while the predicted additive effect of the two stressors was negative, the observed effect of the stressor combination was positive (abundance of large individuals in sediment x insecticide mesocosms was 1.7 times higher than predicted by additivity, Figure 3.4d). Furthermore, imidacloprid attenuated the positive effect of nutrient enrichment on benthic invertebrate richness; nutrient x insecticide tanks had 19% lower taxa richness than expected if the effects of the two stressors were independent (antagonistic interaction, Figure 3.4e).

RDA ordination of benthic invertebrates collected the last day of the experi-
ment indicated that nutrient pulses significantly altered composition of benthic invertebrate communities in the ponds (RDA nutrient term: $P < 0.001$, Figure 3.5b), mainly by reducing the abundance of Orthocladiinae midge larvae, while increasing the abundance of dragonfly larvae from the genus *Sympetrum* and midge larvae from the subfamily Chironominae. According to the RDA, sedimentation and insecticide had only marginally significant effects on benthic invertebrate composition, which were associated with lower densities of *Procloeon* mayflies and tanypodine midges, as well as higher abundance of Chironominae and *Sympetrum* (Figure 3.5b).

### 3.3.4 Biomass distribution among ecological compartments

We used redundancy analysis to examine the impact of the stressors on the distribution of biomass among the trophic compartments we measured (benthic and pelagic) on the last day of the experiment (Figure 3.7a). Unsurprisingly, nutrient enrichment was the only stressor that significantly altered the distribution of biomass among compartments, explaining 27% of the variation in biomass (RDA nutrient term, $P < 0.01$). According to the RDA ordination, nutrient pulses were strongly correlated with increases in zooplankton and phytoplankton biomass, and reductions of benthic invertebrate biomass in the mesocosms. Plots comparing biomass in mesocosms with and without nutrient additions revealed that zooplankton was disproportionally favoured by nutrient enrichment, showing an average 68% increase in biomass (Figure 3.7b). Phytoplankton, which had much less biomass relative to zooplankton in all mesocosms, was also enhanced by nutrient enrichment (83% increase). On the other hand, consumers in the benthic habitat compartment were negatively affected by the enrichment (26% reduction), while benthic primary producers were not strongly affected.

### 3.3.5 Ecosystem function

The stressor treatments had strong independent effects on net ecosystem production (NEP) of the mesocosms throughout the experiment. Nutrient enrichment pro-
duced the quickest and strongest response on NEP, with significant positive effects from the first week after the application of the treatment that became the strongest one week after the second nutrient addition (Figure 3.2e, f). We also observed negative effects of sedimentation starting after the fourth sediment addition (day 22) until the end of the experiment. LME models of the whole time series indicated that overall, nutrient enrichment increased NEP by 50%, while sedimentation decreased it by 14% (Table 3.3, Figure 3.8a). Insecticide pulses also increased average NEP throughout the experiment, but their effects were not statistically significant (9% increase, $P < 0.1$). Nutrient enrichment was the only treatment with significant effects on biochemical oxygen demand (BOD), increasing it by 14% (Figure 3.8b). Contrary to our expectations we found no evidence of significant effects of any of the treatments on leaf decomposition by the end of the experiment (Table 3.3).

3.4 Discussion

Our findings support the hypothesis that environmentally relevant concentrations of imidacloprid, a widely used systemic insecticide, may interact non-additively with nutrient enrichment and sedimentation, two of the most widespread stressors in agricultural landscapes (Allan, 2004). These results are consistent with a growing body of evidence suggesting that additive frameworks may underestimate or overestimate cumulative effects of common stressors on freshwater ecosystems (Shurin et al., 2012; Piggott et al., 2015b; Wagenhoff et al., 2016). Furthermore, our observations suggest that imidacloprid has the potential to alter freshwater ecosystem metabolism, underscoring the importance of monitoring ecosystem function in ecological impact assessments (Young et al., 2008).

3.4.1 Single stressors strongly affected ecosystem function and structure

Table 3.4 presents a summary of our initial hypothesis and their respective outcomes. In agreement with our first hypothesis, the nutrient enrichment had strong positive effects on the pelagic food web and enhanced net ecosystem production.
and biochemical oxygen demand in the mesocosms. However, contrasting with our notion that nutrients would have positive effects across all ecological compartments, we observed mostly negative effects of the enrichment on benthic food webs in our ponds. In benthic food webs, nutrient additions increased taxa richness but shifted community composition towards more tolerant taxa, and reduced benthic invertebrate biomass. We attribute this tendency towards negative effects on the benthic food web to the strong response of the pelagic compartment to the nutrient subsidy. The rapid increase in phytoplankton biomass may have reduced light penetration, thereby limiting periphyton growth and negating the positive effects of the subsidy in the benthos (Scheffer et al., 1993). Other mesocosm and field studies have reported similar responses to nutrient enrichment in lentic ecosystems, where the rapid response of the phytoplankton shades periphyton growth, dampening its response (Brock et al., 1992b). However, these findings contrast with results of a similar mesocosm experiment of longer duration (16 months), where nutrient additions enhanced biomass across all trophic levels (Shurin et al., 2012).

Our results largely supported our second hypothesis predicting negative effects of sedimentation in most ecological compartments of the pond ecosystem. Weekly sediment additions significantly reduced zooplankton biomass and richness, lowered benthic invertebrate abundance, and limited net ecosystem production in the ponds. These observations are consistent with a large body of literature reporting strong negative effects of fine sediment additions in lotic and lentic ecosystems (Knowlton and Jones, 1995; Wood and Armitage, 1997; Horppila and Liljendahl-Nurminen, 2005; Matthaei et al., 2006). Negative effects of sedimentation on primary production can be due to reduced light penetration, physical abrasion, and impairment of substrate for periphyton recruitment (Davies-Colley et al., 1992; Wood and Armitage, 1997). Deleterious effects on benthic invertebrates are generally related to reduced habitat availability, and physical damage of gills and respiratory surfaces (Wood and Armitage, 1997; Allan, 2004). On the other hand, negative effects on zooplankton biomass can be attributed to decreased feeding rates and ingestion of suspended sediment particles that reduces assimilation efficiency of planktonic filter feeders (Hart, 1988; Horppila and Liljendahl-Nurminen, 2005; Rellstab and Spaak, 2007).
Contrary to our third prediction, we did not observe significantly lower densities of benthic invertebrates in mesocosms treated with imidacloprid. However, imidacloprid did cause significant compositional changes on benthic invertebrate communities. Imidacloprid additions were associated with reduced densities of midge larvae from the subfamily Tanypodinae and lower densities of Proclœon, the most abundant Ephemeroptera genus in our pond ecosystems. These changes were similar to those reported by previous outdoor experiments and generally agree with toxicity tests showing that Ephemeroptera, Trichoptera, and Chironomidae species are particularly sensitive to neonicotinoids (Morrissey et al., 2015). For example, Mohr et al. (2012) also reported that imidacloprid pulses in outdoor stream mesocosms reduced densities of Tanypodinae, as well as densities of Baetidae, the dominant Ephemeroptera family in their stream mesocosms. Similarly, Colombo and Mohr (2013) reported that imidacloprid reduced densities of Tanypodinae and Ephemeroptera in outdoor lentic microcosms.

Also contrary to our initial predictions, our results suggested strong negative effects of imidacloprid on zooplankton density. We did not expect lower zooplankton densities at the concentration levels we tested because our zooplankton communities were mostly comprised by cladoceran crustaceans, which are generally considered tolerant to neonicotinoid insecticides (Sánchez-Bayo et al., 2016). For example, a laboratory bioassay reported median lethal concentrations (LC$_{50}$) between 65000-133000 µg L$^{-1}$ after 48 h of static exposure for two common cladoceran species (Sánchez-Bayo and Goka, 2006). We observed significant reductions in zooplankton density, and significant changes in composition after two short pulses of imidacloprid of approximately 1.97 µg L$^{-1}$ in our mesocosms. Interestingly, this is not the first mesocosm study reporting effects on zooplankton at levels that are much lower than those suggested by laboratory toxicity tests. Sánchez-Bayo et al. (2016) noted in their review paper that mesocosm studies consistently report population and community effects of neonicotinoids at concentrations lower than the LC$_{50}$ of the species under study. In this regard, neonicotinoid insecticides differ from other pesticides, which regularly show lower toxicity in realistic field scenarios than in acute toxicity tests (Sánchez-Bayo et al., 2016). This discrepancy is attributed to the irreversible neurotoxicological effects of neonicotinoids that re-
sult in impacts that cumulate over time, which amplifies their effects with repeated exposures (Sánchez-Bayo et al., 2016).

### 3.4.2 Imidacloprid’s indirect effects on ecosystem function

This is one of few studies evaluating ecosystem-scale functional responses to neonicotinoid insecticides (Pestana et al., 2009; Sánchez-Bayo et al., 2016). In agreement with our fifth prediction, our results suggest potential indirect effects of imidacloprid on whole-ecosystem primary production, as the insecticide tended to increase NEP. However, this result was slightly surprising, because we did not observe strong effects of imidacloprid on periphyton and phytoplankton biomass separately. We think this result may be attributed to aggregate indirect impacts of the insecticide on all primary producers in the ponds. Disconnections between patterns observed in algae biomass and NEP have been reported by previous studies (Young et al., 2008), and highlight the complexity of the interactions between different structural components of aquatic ecosystems. More research on impacts of imidacloprid on net ecosystem productivity is desirable to get a better understanding of other potential indirect effects.

On the other hand, our results did not support our sixth hypothesis predicting significant effects of imidacloprid on leaf decomposition. This prediction was based on previous mesocosm and microcosm studies reporting negative indirect effects of the insecticide on leaf decomposition, due to the inhibition of invertebrate shredders (Kreutzweiser et al., 2008; Pestana et al., 2009). However, such studies tested higher concentrations of imidacloprid over longer duration than what was tested in our study. Thus, it is possible that the exposure to imidacloprid evaluated in this experiment was not sufficient to cause inhibitory effects on invertebrate shredders. For example, Kreutzweiser et al. (2008) observed inhibition of leaf processing by invertebrates in microcosms treated with 12 µg L⁻¹ and higher. Another mesocosm experiment evaluating three pulses of similar concentration (1.63 µg L⁻¹) as our two pulses (1.97 µg L⁻¹) also reported inhibition of leaf litter decomposition by invertebrates, but each of their pulses lasted 24 h which likely resulted in higher exposure of the invertebrates to the insecticide compared to our treatment.
3.4.3 Frequent antagonistic interactions between imidacloprid and other agricultural stressors

Our results generally support the hypothesis that imidacloprid can interact with nutrient enrichment and sedimentation at environmentally relevant concentrations. In agreement with our seventh prediction, imidacloprid interacted antagonistically with nutrient enrichment. This finding supports previous investigations documenting antagonistic interactions between moderate concentrations of imidacloprid and high levels of nutrient enrichment (Alexander et al., 2013, 2016). For instance, Alexander et al. (2016) observed that the negative effect of lowest observable effects concentrations of imidacloprid (LOEC: 1.39–1.60 µg L\(^{-1}\)) on the sensitive Baetis mayflies was masked by moderate and high levels of nutrient enrichment on stream microcosms. In our experiment, the insecticide independently did not have significant negative effects on benthic invertebrate richness, but dampened the positive impacts of nutrient enrichment when the two stressors were combined.

Given the tendency of imidacloprid to persist longer in sediment than in water (Van Dijk et al., 2013), our eighth hypothesis predicted synergy between imidacloprid and sediment, through increased contact exposure and ingestion of the insecticide by zooplankton and benthic invertebrates. However, our observations suggest that the combination of the two stressors interacted antagonistically to affect zooplankton communities in the pond mesocosms. We attribute this antagonistic interaction to the fact that the two stressors tended to affect the same sensitive zooplankton genera in the ponds. Thus, their combination caused less taxa losses in the system than predicted by additivity. This observation can be explained by the community co-tolerance hypothesis proposed by Vinebrooke et al. (2004). This hypothesis predicts that when the species’ sensitivities to the stressors are positively correlated, either stressor eliminates certain sensitive species but leaves species that are likely to be tolerant to the second stressor, so there are no further losses in the system. This response is known as stress-induced community tolerance (Vinebrooke et al., 2004), and has been reported by other multiple stressor studies on
This is the first experimental examination of 3-way interactions between imidacloprid, nutrient enrichment, and sedimentation. In keeping with results observed for their 2-way interactions, we observed complex 3-way interactions only on diversity metrics, and they were all antagonistic at the levels tested. These findings further support the hypothesis of stress-induced community tolerance of pelagic and benthic communities to the combined impacts of sedimentation, nutrient enrichment, and imidacloprid.

### 3.4.4 Implications

Mesocosm experiments are a useful tool to isolate stressor impacts while incorporating enough ecological complexity to test indirect effects of the stressors, as well as sufficient replication for rigorous hypothesis testing (Culp et al., 2000; Spivak et al., 2011; Stewart et al., 2013). However, they require the simplification of very complex systems, and are conducted in limited temporal and spatial scales, therefore the extrapolation of our experimental results to field conditions should be done with care. As an example, in our experiment we did not observe significant effects of any of the stressors on leaf decomposition, which generally contrasts with a number of studies reporting direct impacts of nutrient enrichment, and sedimentation, and suggesting potential indirect effects of imidacloprid contamination. The lack of response of leaf decomposition to either of the treatments could be associated with the season when the experiment was conducted (spring). For instance, a previous pond mesocosm study conducted across several seasons, reported that the impacts of nutrient subsidies on decomposition varied with season, with little effects during the spring, but strong effects during the summer (Greig et al., 2012). However, despite the normal limitations, our study still offers novel empirical information about the nature of some multiple stressor interactions, and documents potential whole-ecosystem effects of a popular neonicotinoid insecticide.

Most environmental impact assessment frameworks currently employed to manage anthropogenic stressors on freshwater ecosystems are developed for single...
stressors and are largely focused on stressor characteristics (Crain et al., 2008; Segner et al., 2014; Gessner and Tlili, 2016). These frameworks implicitly assume that multiple stressors interact additively, so their cumulative impacts on a biological system are predicted by the addition of single stressor effects. Our findings support a growing body of literature suggesting that additive frameworks may not be adequate to predict impacts of multiple stressors on freshwater ecosystems (Shurin et al., 2012; Piggott et al., 2015b; Wagenhoff et al., 2016). Furthermore, we observed that the outcome of the multiple stressor interactions was strongly dependent on the correlation between the species’ tolerance to the individual stressors, a finding that fully supports emerging environmental assessment frameworks that increasingly focus on properties of biological receptors, rather than properties of stressors (e.g. Rohr et al., 2006; Segner et al., 2014).

Our study also supports the notion that field mesocosm experiments should be used to complement laboratory bioassays, in order to assess risk of toxicants on freshwater ecosystems (Colombo and Mohr, 2013; Alexander et al., 2016). Not only did we detect negative effects of imidacloprid on zooplankton at concentrations lower than the LC$_{50}$ of several common species in the community, but we also observed previously unreported effects of the insecticide on ecosystem functions. Furthermore, our results showed the complexity of predicting impacts on ecosystem function based on changes observed on ecosystem structure, further supporting previous investigations suggesting that functional metrics should be implemented in routine ecological assessments, in order to obtain a reliable evaluation of ecosystem health (Gessner and Chauvet, 2002; Young et al., 2008; Piggott et al., 2015a).
Table 3.1: ANOVA summary of linear models evaluating impacts of the stressor treatments on water quality and habitat characteristics of the freshwater mesocosms. Significant effects ($P < 0.05$) are indicated in bold.

<table>
<thead>
<tr>
<th>Response variables</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
<th>$F$</th>
<th>$P$</th>
<th>$F$</th>
<th>$P$</th>
<th>$F$</th>
<th>$P$</th>
<th>$F$</th>
<th>$P$</th>
<th>$F$</th>
<th>$P$</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{PO}_4\text{-P}$</td>
<td>1, 24</td>
<td>235.125</td>
<td>$&lt; 0.001$</td>
<td>0.185</td>
<td>0.671</td>
<td>0.400</td>
<td>0.533</td>
<td>0.005</td>
<td>0.944</td>
<td>0.613</td>
<td>0.441</td>
<td>0.877</td>
<td>0.358</td>
<td>1.676</td>
<td>0.208</td>
</tr>
<tr>
<td>$\text{NH}_4\text{-N}$</td>
<td>1, 24</td>
<td>1758.047</td>
<td>$&lt; 0.001$</td>
<td>0.139</td>
<td>0.685</td>
<td>0.416</td>
<td>5.192</td>
<td>$0.032$</td>
<td>0.927</td>
<td>0.026</td>
<td>0.872</td>
<td>$&lt; 0.001$</td>
<td>0.986</td>
<td>1.716</td>
<td>0.203</td>
</tr>
<tr>
<td>$\text{NO}_3\text{-N}$</td>
<td>1, 24</td>
<td>2.345</td>
<td>0.139</td>
<td>0.685</td>
<td>0.416</td>
<td>5.192</td>
<td>$0.032$</td>
<td>0.927</td>
<td>0.026</td>
<td>0.872</td>
<td>$&lt; 0.001$</td>
<td>0.986</td>
<td>1.716</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td>DIP:DIN</td>
<td>1, 24</td>
<td>0.115</td>
<td>0.737</td>
<td>0.561</td>
<td>0.461</td>
<td>4.095</td>
<td>0.054</td>
<td>$&lt; 0.001$</td>
<td>0.992</td>
<td>0.092</td>
<td>0.764</td>
<td>0.028</td>
<td>0.868</td>
<td>0.282</td>
<td>0.600</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>1, 24</td>
<td>35.519</td>
<td>$&lt; 0.001$</td>
<td>0.251</td>
<td>0.621</td>
<td>3.789</td>
<td>0.063</td>
<td>0.880</td>
<td>0.357</td>
<td>0.001</td>
<td>0.973</td>
<td>0.481</td>
<td>0.495</td>
<td>3.177</td>
<td>0.087</td>
</tr>
<tr>
<td>Conductivity</td>
<td>1, 24</td>
<td>1.211</td>
<td>0.282</td>
<td>0.137</td>
<td>0.714</td>
<td>1.234</td>
<td>0.278</td>
<td>0.042</td>
<td>0.839</td>
<td>0.045</td>
<td>0.834</td>
<td>0.208</td>
<td>0.653</td>
<td>0.058</td>
<td>0.812</td>
</tr>
<tr>
<td>pH</td>
<td>1, 24</td>
<td>36.899</td>
<td>$&lt; 0.001$</td>
<td>0.012</td>
<td>0.915</td>
<td>9.999</td>
<td>$0.004$</td>
<td>0.022</td>
<td>0.884</td>
<td>0.062</td>
<td>0.805</td>
<td>0.012</td>
<td>0.915</td>
<td>1.513</td>
<td>0.231</td>
</tr>
<tr>
<td>Turbidity</td>
<td>1, 24</td>
<td>1.202</td>
<td>0.284</td>
<td>256.721</td>
<td>$&lt; 0.001$</td>
<td>0.603</td>
<td>0.445</td>
<td>0.921</td>
<td>0.347</td>
<td>0.235</td>
<td>0.632</td>
<td>1.551</td>
<td>0.225</td>
<td>0.008</td>
<td>0.930</td>
</tr>
<tr>
<td>Sedimentation</td>
<td>1, 24</td>
<td>0.300</td>
<td>0.589</td>
<td>97.404</td>
<td>$&lt; 0.001$</td>
<td>0.700</td>
<td>0.411</td>
<td>1.167</td>
<td>0.291</td>
<td>0.993</td>
<td>0.329</td>
<td>0.258</td>
<td>0.616</td>
<td>1.848</td>
<td>0.187</td>
</tr>
</tbody>
</table>
**Table 3.2:** Average imidacloprid concentration measured in all tanks treated with the insecticide, and three randomly selected insecticide controls (tanks not treated with the insecticide), on days 1 and 35 of the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1 (± SE)</th>
<th>Day 35 (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insecticide control</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>I</td>
<td>1.985 (0.2)</td>
<td>0.071 (0.01)</td>
</tr>
<tr>
<td>N*I</td>
<td>2.038 (0.05)</td>
<td>0.094 (0.01)</td>
</tr>
<tr>
<td>S*I</td>
<td>1.943 (0.06)</td>
<td>0.04 (0.01)</td>
</tr>
<tr>
<td>N<em>S</em>I</td>
<td>1.916 (0.06)</td>
<td>0.034 (0.01)</td>
</tr>
<tr>
<td>Mean insecticide tanks</td>
<td>1.97 (0.01)</td>
<td>0.06 (0.001)</td>
</tr>
</tbody>
</table>
Table 3.3: ANOVA summary of linear models evaluating impacts of the stressor treatments on habitat, benthic invertebrates, zooplankton, and ecosystem function throughout the experiment. Significant effects ($P < 0.05$) are indicated in bold.

<table>
<thead>
<tr>
<th>Response variables</th>
<th>df</th>
<th>$N$</th>
<th>$S$</th>
<th>$I$</th>
<th>$N \times S$</th>
<th>$N \times I$</th>
<th>$S \times I$</th>
<th>$N \times S \times I$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pelagic community</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytoplankton Chl-a</td>
<td>1, 24</td>
<td>15.223</td>
<td>0.001</td>
<td>0.001</td>
<td>0.794</td>
<td>0.382</td>
<td>0.047</td>
<td>0.831</td>
</tr>
<tr>
<td>Zooplankton density</td>
<td>1, 24</td>
<td>2.102</td>
<td>0.160</td>
<td>2.463</td>
<td>0.130</td>
<td>7.668</td>
<td><strong>0.011</strong></td>
<td>0.001</td>
</tr>
<tr>
<td>Zooplankton biomass</td>
<td>1, 24</td>
<td>27.188</td>
<td><strong>&lt;0.001</strong></td>
<td>4.961</td>
<td><strong>0.036</strong></td>
<td>0.216</td>
<td>0.647</td>
<td>0.076</td>
</tr>
<tr>
<td>Zooplankton richness</td>
<td>1, 24</td>
<td>1.233</td>
<td>0.278</td>
<td>5.842</td>
<td><strong>0.024</strong></td>
<td>0.107</td>
<td>0.747</td>
<td>0.346</td>
</tr>
<tr>
<td>Zooplankton Shannon-Wiener diversity</td>
<td>1, 24</td>
<td>2.897</td>
<td>0.102</td>
<td>1.968</td>
<td>0.173</td>
<td>0.031</td>
<td>0.862</td>
<td>0.006</td>
</tr>
<tr>
<td>Zooplankton evenness</td>
<td>1, 24</td>
<td>2.604</td>
<td>0.120</td>
<td>0.374</td>
<td>0.547</td>
<td>0.029</td>
<td>0.866</td>
<td>0.073</td>
</tr>
<tr>
<td><strong>Benthic community</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periphyton Chl-a</td>
<td>1, 24</td>
<td>2.032</td>
<td>0.167</td>
<td>0.840</td>
<td>0.369</td>
<td>0.133</td>
<td>0.719</td>
<td>0.257</td>
</tr>
<tr>
<td>Abundance</td>
<td>1, 24</td>
<td>0.018</td>
<td>0.894</td>
<td>9.158</td>
<td><strong>0.005</strong></td>
<td>2.539</td>
<td>0.124</td>
<td>3.260</td>
</tr>
<tr>
<td>Biomass</td>
<td>1, 24</td>
<td>0.324</td>
<td>0.574</td>
<td>1.303</td>
<td>0.265</td>
<td>0.244</td>
<td>0.626</td>
<td>1.141</td>
</tr>
<tr>
<td>Mean size</td>
<td>1, 24</td>
<td>0.128</td>
<td>0.723</td>
<td>1.899</td>
<td>0.181</td>
<td>1.664</td>
<td>0.209</td>
<td>2.678</td>
</tr>
<tr>
<td>Abundance small size</td>
<td>1, 24</td>
<td>0.007</td>
<td>0.935</td>
<td>8.398</td>
<td><strong>0.008</strong></td>
<td>1.259</td>
<td>0.273</td>
<td>1.635</td>
</tr>
<tr>
<td>Abundance medium size</td>
<td>1, 24</td>
<td>0.153</td>
<td>0.699</td>
<td>7.551</td>
<td><strong>0.011</strong></td>
<td>3.971</td>
<td>0.058</td>
<td>3.562</td>
</tr>
<tr>
<td>Abundance large size</td>
<td>1, 24</td>
<td>0.316</td>
<td>0.579</td>
<td>1.052</td>
<td>0.315</td>
<td>0.202</td>
<td>0.657</td>
<td>0.166</td>
</tr>
<tr>
<td>Richness</td>
<td>1, 24</td>
<td>9.328</td>
<td><strong>0.005</strong></td>
<td>3.358</td>
<td>0.079</td>
<td>0.731</td>
<td>0.401</td>
<td>1.209</td>
</tr>
<tr>
<td>Shannon-Wiener diversity</td>
<td>1, 24</td>
<td>1.496</td>
<td>0.233</td>
<td>1.386</td>
<td>0.251</td>
<td>0.142</td>
<td>0.709</td>
<td>0.166</td>
</tr>
<tr>
<td>Evenness</td>
<td>1, 24</td>
<td>0.372</td>
<td>0.548</td>
<td>0.087</td>
<td>0.770</td>
<td>0.019</td>
<td>0.892</td>
<td>1.636</td>
</tr>
<tr>
<td><strong>Ecosystem function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf decomposition</td>
<td>1, 24</td>
<td>0.241</td>
<td>0.628</td>
<td>1.100</td>
<td>0.305</td>
<td>0.085</td>
<td>0.773</td>
<td>1.100</td>
</tr>
<tr>
<td>Net ecosystem production</td>
<td>1, 24</td>
<td>68.869</td>
<td><strong>&lt;0.001</strong></td>
<td>8.835</td>
<td><strong>0.007</strong></td>
<td>3.462</td>
<td>0.075</td>
<td>0.058</td>
</tr>
<tr>
<td>Biochemical oxygen demand</td>
<td>1, 24</td>
<td>9.765</td>
<td><strong>0.005</strong></td>
<td>1.367</td>
<td>0.254</td>
<td>0.003</td>
<td>0.957</td>
<td>0.180</td>
</tr>
</tbody>
</table>
### Table 3.4: Overview of hypotheses and study results

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Stressor</th>
<th>Biological response</th>
<th>Hypothesized effect/interaction</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nutrients</td>
<td>Leaf decomposition, primary production, invertebrate biomass</td>
<td>Positive</td>
<td>Accepted for pelagic compartment, not accepted for benthic</td>
</tr>
<tr>
<td>2</td>
<td>Sediment</td>
<td>Most community metrics and ecosystem processes</td>
<td>Negative</td>
<td>Accepted</td>
</tr>
<tr>
<td>3</td>
<td>Imidacloprid</td>
<td>Benthic invertebrate density</td>
<td>Negative</td>
<td>Not accepted, but observed significant changes in composition</td>
</tr>
<tr>
<td>4</td>
<td>Imidacloprid</td>
<td>Zooplankton density</td>
<td>No effect</td>
<td>Not accepted, observed significant reductions at lower concentrations than expected</td>
</tr>
<tr>
<td>5</td>
<td>Imidacloprid</td>
<td>Net ecosystem productivity</td>
<td>Positive</td>
<td>Marginally significant positive effects</td>
</tr>
<tr>
<td>6</td>
<td>Imidacloprid</td>
<td>Leaf decomposition</td>
<td>Negative</td>
<td>Not accepted</td>
</tr>
<tr>
<td>7</td>
<td>Imidacloprid + Nutrients</td>
<td>Pelagic and benthic invertebrate metrics</td>
<td>Antagonistic</td>
<td>Accepted</td>
</tr>
<tr>
<td>8</td>
<td>Imidacloprid + Sediment</td>
<td>Pelagic and benthic invertebrate metrics</td>
<td>Synergistic</td>
<td>Not accepted, observed mostly antagonistic interactions</td>
</tr>
</tbody>
</table>
Figure 3.1: Photo of the experimental set-up consisting in 32 outdoor freshwater mesocosms located at the University of British Columbia’s experimental pond facility, Vancouver, Canada.
Figure 3.2: Temporal dynamics of phytoplankton biomass (a, b), zooplankton biomass (c, d), and net ecosystem production (e, f) in freshwater mesocosms exposed to nutrient, sediment, and insecticide pulses. Each point represents the mean (± SE) for each treatment (n = 4). Letters are used to represent stressor treatments (N, nutrients; S, sediment; I, insecticide, N+S, nutrient + sediment; N+I, nutrient + insecticide; S+I, sediment + insecticide; N+S+I; nutrient + sediment + insecticide). Dashed vertical lines indicate the dates for nutrient and insecticide additions, while dotted vertical lines indicate sediment additions. Points are slightly jittered to ease interpretability.
Figure 3.3: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant stressor main and interactive effects on zooplankton communities. Let-
ers in the y-axis represent main effects (N, nutrients; S, sediment; I, insecticide) and interaction terms (N*S, nutrient x sediment; N*I, nutrient x insecticide; S*I, sediment x insecticide; N*S*I; nutrient x sed-
iment x insecticide). For main effects positive values indicate increases
in the response variable whereas negative values indicate the opposite.
For interactions, confidence intervals overlapping zero indicate additive
interactions, positive values indicate synergies, and negative values are
antagonisms or reversals (reversals are marked with an R). Symbols
are used to represent significance of the effect according to the linear
mixed effects models presented in Table 3.3: ***$P < 0.001$, **$P < 0.01$,
* $P < 0.05$, $P < 0.1$.
**Figure 3.4:** Bar plots illustrating significant interactive effects of the stressors on zooplankton (a-c) and benthic invertebrate (d-e) metrics. Letter notation for the treatments is consistent with Figure 3.2. Dotted horizontal lines are used to represent the predicted additive effect of the stressors. All shown interactions were classified as antagonistic, except the S*I interaction on abundance of large-sized benthic invertebrates (d) which was a reversal. Bars represent the mean of four replicates (± SE).
Figure 3.5: Redundancy analysis plot showing the effects of the stressor pulses on zooplankton (a) and benthic invertebrate (b) community composition by the end of the experiment. Only the most abundant taxa are labeled. Solid arrows indicate significant main effects ($P < 0.05$) and dashed arrows indicate marginally significant effects ($P < 0.1$), following a permutation test of the full model with 999 randomizations. Letter notation for the treatments is consistent with Figure 3.3. The treatments together explained 35% of the variation on zooplankton composition and 33% of variation on benthic invertebrate composition.
Figure 3.6: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant stressor main and interactive effects on benthic invertebrate communities. Letter notation for the treatments and interpretation of effects is consistent with Figure 3.3. Symbols are used to represent significance of the effect according to the linear fixed effects models presented in Table 3.3: $**P < 0.01$, $*P < 0.05$, $P < 0.1$. 

Figure 3.6: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant stressor main and interactive effects on benthic invertebrate communities. Letter notation for the treatments and interpretation of effects is consistent with Figure 3.3. Symbols are used to represent significance of the effect according to the linear fixed effects models presented in Table 3.3: $**P < 0.01$, $*P < 0.05$, $P < 0.1$. 

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Figure 3.7: Redundancy analysis plot showing the effects of the stressor pulses on the distribution of biomass among food web components in the mesocosms by the end of the experiment (day 36). Solid arrows indicate significant main effects ($P < 0.01$), following a permutation test of the full model with 999 randomizations. Letter notation for the treatments is consistent with Figure 3.3. The treatments together explained 27% of the variation on biomass (a). Barplot comparing average biomass ($n = 16$) of food web components among nutrient treatment levels (b).
Figure 3.8: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant stressor main and interactive effects on ecosystem function. Letter notation for the treatments and interpretation of effects is consistent with Figure 3.3. Symbols are used to represent significance of the effect according to the linear mixed effects models presented in Table 3.3: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, .$P < 0.1$. 

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Chapter 4

Multiple-stressor interactions in tributaries alter downstream ecosystems in stream mesocosm networks

4.1 Introduction

River systems are dendritic networks in which contributing streams join into the tributaries of the main river. This pattern follows a hierarchical configuration, increasing in size and decreasing in number in the downstream direction (Benda et al., 2004; Campbell Grant et al., 2007). Thus, every large river basin is composed of nested subcatchments that have a longitudinal connection through the unidirectional flow of water (Fisher, 1997; Allan and Castillo, 2007; Campbell Grant et al., 2007). The connectivity imposed by the unidirectional flow and the spatial arrangement of river networks, greatly influences ecological dynamics, as subsidies of energy, nutrients, and organic materials are constantly transported across interconnected stream reaches (Gomi et al., 2002; Campbell Grant et al., 2007; Brown...
et al., 2011). This interconnected set of habitats are the reason why river networks are considered meta-ecosystems (Loreau et al., 2003; Brown et al., 2011). In river meta-ecosystems, local community composition, habitat condition, and ecosystem services cannot be understood by focusing solely on local-scale processes, because they are also influenced by processes operating in upstream reaches at the regional scale (Campbell Grant et al., 2007; Brown et al., 2011; Tomscha et al., 2017).

Land-use practices can dramatically alter the input of materials into headwater streams, subsequently affecting subsidies to downstream ecosystems (Gomi et al., 2002; Wipfli et al., 2007). For instance, agricultural practices often increase inputs of fine sediment and nutrients into stream ecosystems (Allan, 2004; Riseng et al., 2011). Numerous studies have demonstrated that nutrient enrichment generally increases standing stocks and processing rates of basal resources in aquatic ecosystems, causing bottom-up effects in aquatic food webs at moderate levels of enrichment, and negative effects at higher levels (Allan, 2004; Woodward et al., 2012b; Wagenhoff et al., 2013). On the other hand, fine sediment inputs are reported to have strong negative effects on stream communities, reducing primary production due to increased turbidity, smothering, and abrasion, filling interstitial spaces for benthic invertebrates, and slowing down organic matter processing (Wood and Armitage, 1997; Matthaei et al., 2006). Furthermore, recent experimental investigations have demonstrated that sedimentation and nutrient enrichment often interact synergistically, causing cumulative effects that are more detrimental than predicted on the basis of the stressors’ individual effects (Matthaei et al., 2010; Piggott et al., 2012; Wagenhoff et al., 2012).

The degradation of headwater watersheds with these and other ecological stressors may cause alterations to downstream hydrology, water quality, geomorphic processes, and even biota, as food resource subsidies coming from headwater ecosystems are disrupted (Freeman et al., 2007). The transmission of disturbances (e.g., fine sediment, nutrients, and other contaminants) from headwaters to downstream ecosystems is governed by a complex array of routing processes taking place at the scale of headwater systems (storage, transformation, and disturbance mechanism), and network systems (synchronized or desynchronized outflows from headwater basins, basin size, basin shape, drainage density, and network geometry) (Gomi
et al., 2002; Wipfli et al., 2007). Empirical evidence regarding the interaction of processes happening at multiple scales is necessary to develop predictive understanding about the consequences of cumulative headwater degradation. However, studies addressing cumulative effects on river systems often focus on specific sections of the river network, either evaluating responses at the headwater scale or at the network scale (Rasmussen et al., 2012; Schneider et al., 2013). To date few studies explicitly integrate both scales to measure how processes in individual headwater systems interact to affect inputs of material and function on downstream ecosystems (but see Patrick and Fernandez, 2013).

Here we present the results of a mesocosm experiment designed to study how interactions among multiple stressors within tributaries may affect downstream ecosystem function, diversity, and physical habitat. Using a mesocosm model of a stream network, we manipulated sediment and nutrient levels in the tributaries of second-order channels, to determine individual and combined effects of disturbances on tributaries and recipient ecosystems. We chose this stressor combination as they offered a good model to study cumulative effects of headwater degradation at the network scale. First, as mentioned earlier, there is already empirical knowledge about their potential non-additive interactions on stream ecosystems (e.g., Matthaei et al., 2010; Piggott et al., 2012; Wagenhoff et al., 2012). Second, they may have different rates of delivery from headwaters to downstream ecosystems, due to different storage and transformation mechanisms within headwaters (Bernhardt et al., 2005; Wipfli et al., 2007).

Our treatments were designed to test: i) individual and combined effects of nutrient enrichment and sedimentation on ecological structure and function of tributary streams; ii) the potential effect of complex stressor interactions within the tributaries on recipient second-order channels; and iii) how the level of disturbance within the tributaries affects ecological function and structure of recipient downstream ecosystems. Our overarching hypothesis was that stressor additions in tributary streams would have detectable effects on the structure and function of downstream recipient ecosystems. Specifically, for tributary channels we hypothesized that: 1) nutrient additions would have positive effects on most biological responses; 2) sediment additions would have negative effects on most biological
responses; and 3) there would be complex non-additive interactions between nutrient enrichment and sedimentation. For second-order channels we hypothesized that: 4) complex multiple-stressor interactions within the tributaries would influence responses of recipient downstream ecosystems; and that 5) increasing levels of disturbance in the tributaries would cause proportional increases of disturbance on downstream ecosystems.

4.2 Methods

4.2.1 Experimental design

We built a network of 36 stream mesocosm channels in the Malcolm Knapp Research Forest of the University of British Columbia, near Maple Ridge, British Columbia, Canada (49° 16 N, 122° 34 W; Figure 4.1). In the design, 24 mesocosms, which will be referred to as “first-order” channels, converged downstream in pairs to form 12 “second-order” channels (channel dimensions: 6.8 m x 0.15 m, Figure 4.2). All channels were stocked with a 4 cm layer of washed, mixed gravels (0.5 - 3.0 cm grain size range) and were continuously gravity-fed with water from Mayfly Creek, an adjacent oligotrophic stream which is described in detail in Richardson (1991). Water from Mayfly Creek was distributed from two header boxes with outflow valves that controlled water flow, set to about 1 L s⁻¹ to each channel throughout the experiment. Average slope was 0.06 m m⁻¹ for the first-order channels and 0.03 m m⁻¹ for the second order channels. Four weeks prior the beginning of the experiment, we allowed immigration of invertebrates from Mayfly Creek to colonize the channels via drift. As this method has proven appropriate to obtain consistent invertebrate densities in previous experiments at the same location (Lecerf and Richardson, 2011; Richardson, 1991), we did not collect premanipulation samples for this study.

Our 22-day experimental manipulation was conducted in the fall season, from November 7 to November 28 of 2013. We manipulated fine sediment and nutrients in the 24 first-order channels on day 1 of the experiment. We evaluated two levels
of each stressor (added, ambient) in the following combinations (Figure 4.2): i) ambient levels of sediment and nutrients (control treatment, \( n = 12 \)); ii) added nutrients and ambient levels of sediment (N treatment, \( n = 4 \)); iii) added sediment and ambient levels of nutrients (S treatment, \( n = 4 \)); and iv) added nutrients and added sediment (N+S treatment, \( n = 12 \)). High levels of deposited sediment (approximately 60% stream bed coverage) were achieved by adding 3 L of sand (< 0.5 mm, “medium sand” on the Wentworth scale) as evenly as possible to each sediment-addition first-order channel. These sedimentation levels are equivalent to values reported in rivers affected by agricultural practices (Townsend et al., 2008; Wagenhoff et al., 2012), and are similar to those used in several experiments evaluating the effects of sedimentation on stream ecosystems (e.g., Matthaei et al., 2010; Louhi et al., 2017). We used 4-month, slow-release fertilizer pellets (Florikote, NPK: 15-5-15), to achieve continuous nutrient enrichment throughout the experiment in nutrient addition channels. We added 14 g of Florikote to each treated channel for a target phosphorus concentration of 3 \( \mu g \) L\(^{-1} \) above background nutrient levels (approximate background concentrations: 3.7 \( \mu g \) P-PO\(_4\) L\(^{-1} \), 123.4 \( \mu g \) dissolved inorganic nitrogen L\(^{-1} \), and 74.5 N:P ratio; García et al., 2017). This enrichment level is equivalent to nutrient additions that have been previously reported to cause significant responses in experimental channels fed by Mayfly Creek (Kiffney and Richardson, 2001).

Treatments on first-order channels were assigned so each second-order channel was exposed to one of the following four tributary treatments (Figure 4.2): a) two control tributaries; b) one control tributary and one tributary with both stressors; c) nutrients added in one tributary and sediment in the other; and d) nutrients and sediment simultaneously added in both tributaries. Each tributary treatment had three replicates for a total of 12 second-order channels.

### 4.2.2 Response variables

We measured variables reflecting the effect of the treatments on habitat condition (sedimentation rates, water nutrient concentrations), benthic invertebrate communities (density, functional feeding groups, drift rates), and ecosystem function (leaf
decomposition) in first- and second-order channels. Water samples were collected on day 22 to determine nutrient concentrations (PO$_4$-P, NH$_4$-N, and NO$_3$-N) in the second-order channels, and were analyzed by Maxxam Analytics, Burnaby, British Columbia, using standard methods (APHA, 2005). To quantify sedimentation rates in the channels, we collected all the substrate present in three random quadrants (0.1 m x 0.8 m) of each first- and second-order channel. Substrate collection was carried out using a small D-net built to fit in the channels. We placed the d-net downstream from the sample quadrant, and disturbed the area within the quadrant until all the substrate was collected in the d-net (64 µm mesh size). The collected substrate was filtered through a 2 mm sieve stacked on top of a 0.5 mm sieve to separate the gravel. The filtered sand was stored in sealed plastic buckets and transported to the laboratory. In the laboratory the contents of each bucket were oven-dried at 40°C for 10 days, weighed, ashed at 500°C for three hours, and reweighed to quantify ash-free dry mass (AFDM).

We collected invertebrate drift from all the experimental channels twice, once near the beginning of the experiment (day 2) and once at the end of the experiment (day 22). Drift samples were collected by placing 250 µm mesh nets at the end of each channel. Second-order channels were sampled for 24 h periods, whereas first-order channels were sampled for 2 h periods (10 am to 12 pm) to avoid disrupting the transport of materials to second-order channels. All drift samples were preserved in 80% ethanol and transported to the laboratory. We sampled benthic invertebrate communities in all the experimental channels once, at the end of the experiment (day 22). One composite invertebrate sample was collected in each channel using a small Surber sampler (0.017 m$^2$, 250 µm mesh size) in four random locations (total sampled area in each channel: 0.068 m$^2$). Composite Surber samples were stored in 80% ethanol and sorted later under the dissecting microscope for invertebrates. All benthic invertebrates were enumerated, identified to the lowest practical taxonomic level (usually genus), and classified into functional feeding groups according to Merritt and Cummins (1996). We computed 11 invertebrate variables for each first- and second-order channel: 1) total invertebrate density; 2) total taxa richness; 3) Shannon-Wiener diversity; 4) density of Ephemeroptera, Plecoptera, and Trichoptera (EPT) taxa; 5) richness of EPT taxa; 6) density of
scrapers; 7) shredders; 8) predators; and 9) collectors; 10) drift rate (total number of individuals drifting per hour); and 11) EPT drift flux (number of EPT drifting per sampling period).

To measure leaf decomposition, we introduced one leaf pack of red alder leaves in coarse-mesh bags (3 g air-dry weight; 10 mm mesh) into each channel one day before treatment application (day 0). On day 22, leaf packs were removed from the channels and stored at -18°C until processed in the laboratory to estimate remaining AFDM, according to standard methods (Hauer and Lamberti, 2007). Posterior processing involved defrosting, rinsing, and drying at 60°C for 5 days. Remaining leaf material was then weighed, ashed at 500°C, and reweighed to calculate AFDM.

4.2.3 Data analysis

In order to test our first three hypotheses, we used linear fixed-effects models to quantify individual and combined effects of the stressors on response variables recorded in the first-order channels. For each response variable the model tested was: \( y = b_0 + b_1N + b_2S + b_3N \times S \), where \( N \) is the nutrient treatment and \( S \) the sediment treatment. Significance levels for all our tests was \( P < 0.05 \) and was evaluated with ANOVAs. Following the recommendation of Nakagawa and Cuthill (2007) we present standardized effect size estimates for all findings with \( P < 0.1 \), so readers can judge the biological importance of the results. Hedges’ \( d \) estimates of effect size (Gurevitch and Hedges, 2006) were calculated from the \( t \) values of our linear models using the formulas provided by Nakagawa and Cuthill (2007). In order to improve the graphical representation of our results, we coded significant main effects to represent the direction of the response of manipulated versus control mesocosms (i.e. positive effect sizes indicate increases in the response variable, while negative effect sizes indicate the opposite). Further, we coded 2-way interaction effect sizes to represent the classification of the interaction according to the framework proposed by Jackson et al. (2016). Thus, positive interaction effect sizes represent synergistic interactions (i.e. the combined effect of the stressors is greater than the sum of their individual effects), whereas negative effect sizes
represent either antagonistic interactions (i.e. the combined effect of the stressors is less than the sum of their individual effects) or reversal interactions (i.e. the combined effect of the stressors is in the opposite direction than the sum of the individual effects). Given that the counts of EPT taxa drifting out of the first-order channels were low and contained many zeros, we could not apply a linear model to this variable. Instead, we used a zero-inflated Poisson (ZIP) regression. The ZIP model has two components: a Poisson model with log link (count model) that evaluates the effect of the explanatory variables on the counts; and a negative binomial with logit link model (zero model) that evaluates the effect of the explanatory variables on the probability of zero counts in the data.

Data recorded in the second-order channels were divided into two subsets to test our fourth and fifth hypotheses. For our fourth hypothesis, we used data corresponding to tributary treatments b and c (Figure 4.2) and compared the two treatments using independent t-tests. Thus, we compared whether applying both nutrients and sediment in the same tributary (cumulative effect; tributary treatment b) had the same effect downstream as applying nutrients in one tributary and sediment in the other tributary (single effect: tributary treatment c). For our fifth hypothesis, we used data corresponding to tributary treatments a, b, and d to evaluate the effect of tributary disturbance level on recipient second-order channels (Figure 4.2). We excluded tributary treatment c from this analysis in order to avoid potential confounding effects of multiple-stressor interactions within the tributaries. This way we ensured we were able to test a linear increase in tributary disturbance. We used single linear regressions of the form: \( y = b_0 + b_1x_1 \), where \( x_1 \) was a 3-level factor representing the degree of disturbance in the first-order tributaries (i.e. the number of tributaries treated with the two stressors). The three levels included: 0) a control with no stressors in the tributaries; 1) only one tributary with both stressors; and 2) two tributaries with both stressors (tributary treatments a, b, and d in Figure 4.2, respectively).
4.3 Results

4.3.1 Stressor effects on first-order channels

The sediment treatment caused significantly higher sedimentation rates in the first-order channels (Table 4.1). By the end of the experiment, first-order channels treated with sediment had on average 61 times more sediment accumulated in the substrate (1821.7 ± 312.8 g m⁻²) than untreated channels (29.8 ± 13.8 g m⁻²). Sediment additions also increased the absolute amount of organic sediment deposited (sediment AFDM) by 2.6 times (Table 4.1). However, on average, sediment deposited in these channels had significantly lower organic content (organic content sediment treatment: 0.6% ± 0.15%, mean ±SE) than sediment in the remaining first-order channels (organic content: 30% ± 6%, Table 4.1).

We detected significant negative effects of the sediment treatment on total invertebrate density in the first-order channels (Table 4.1). Channels treated with sediment had on average 34% fewer individuals than channels without sediment (Figure 4.3a). According to our findings, this reduction was associated with negative impacts on invertebrate predators ($P = 0.005$, 55% reduction) and, to a lesser extent, collectors ($P = 0.07$, 46% reduction). In terms of ecosystem functioning, we observed a significant nutrient x sediment interaction affecting leaf decomposition in the first-order channels (Figure 4.4). This interaction was classified as an antagonism; while both stressors tended to have positive effects on leaf decomposition independently (the individual effect of sediment was significant, while the individual effect of nutrients was not), nutrient x sediment channels had similar decomposition rates as the control treatments (Figure 4.4). In other words, leaf packs in nutrient x sediment channels lost in average 25% less ash free dry mass than expected if the effect of the two stressors was additive.

Nutrient and sediment additions did not significantly impact the total number of invertebrates drifting per hour on days 2 and 22 after treatment application. However, we observed significant effects of both treatments on drift flux of individuals from the orders Ephemeroptera, Plecoptera, and Trichoptera on day 22 (Table 4.2).
According to the zero-inflated Poisson regression, nutrient and sediment additions did not influence the probability of excess zeros on the 2-hour drift samples (Figure 4.5a), but had strong effects on the counts of EPT drifting from the channels (Figure 4.5b). Nutrient additions had a negative effect on EPT drift flux, as there were 90% fewer individuals from these orders drifting from channels treated with nutrients. On the other hand, sediment had the opposite effect; sediment-channels had twice as many EPT drifting as the other first-order channels (91% increase in drift flux).

4.3.2 Stressor effects on second-order channels

The t-tests used to compare the impact of applying both stressors in the same tributary versus the impact of applying them in different tributaries (hypothesis 4) indicated that there were no significant differences between sediment deposition rates and nitrate concentration between the two tributary treatments (Table 4.3). Unfortunately, phosphorous concentrations were under detection levels (5 µg L\(^{-1}\)) in all water samples, so we could not determine whether there were significant differences for this element. However, we observed that EPT density and EPT richness were significantly different among the two treatments. There was on average 38% higher density and 45% higher richness of EPT taxa in second-order channels fed by tributaries where the stressors were applied separately (single treatment), when compared to channels fed by tributaries where the stressors were applied simultaneously (cumulative treatment).

Linear models evaluating response variables in the second-order channels as a function of disturbance level in the tributaries (hypothesis 5, Table 4.4), indicated that sediment dry mass (total sediment, 4.6a) and sediment ash-free dry mass (organic sediment, 4.6b) significantly increased with the level of disturbance to the tributaries. According to Tukey’s HSD post-hoc test, total and organic sediment deposition in the maximum level of disturbance (level 2) were significantly higher than sediment deposition in the control and the first level of disturbance (Figure 4.6a-b). However, even at the highest level of tributary disturbance, average sediment deposition in second order channels (351.6 ± 93.84 g m\(^{-2}\), mean ± SD) was
much lower than deposition in first order tributaries treated with sediment (1821.7 ± 1083.8 g m⁻²). On the other hand, we did not detect a significant relationship between tributary level of disturbance and the concentration of nitrate in second-order channels (Table 4.4). In terms of the biological responses, the only variable that significantly responded to the tributary disturbance was EPT density (Table 4.4). According to our observations, EPT density tended to increase with increasing level of disturbance (Figure 4.6c). Tukey’s HSD tests indicated that EPT density in the highest level of tributary disturbance (both tributaries treated with both stressors) was significantly higher than EPT density in the control second-order channels (both tributaries with natural levels of nutrients and sediment).

4.4 Discussion

Our results generally supported the hypothesis that upstream disturbance can influence ecological function and structure of downstream recipient ecosystems in a stream network. However, most of the downstream effects we observed in our study did not support our initial predictions. Due to the small scale of our experimental stream network, our hypotheses were based on the assumption that tributary treatments would affect downstream ecosystems mostly through the transmission of disturbances, or the movement of sediment and nutrients from tributaries to second-order channels. Counter to this assumption, most downstream impacts in our study were a result of within network dispersal of sensitive taxa, as a response to stressor additions in the tributaries.

4.4.1 Stressors altered invertebrate communities and ecosystem function in first-order channels

In our first hypothesis, we predicted that nutrient additions would enhance primary production and organic matter decomposition in our experimental systems, exerting positive bottom-up effects on invertebrate communities (Woodward et al., 2012b; Rosemond et al., 2015). However, we did not detect significant individual effects of the nutrient additions on invertebrate density or leaf decomposition
rates. The only variable that was significantly affected by nutrient additions was EPT drift flux, which was strongly depressed by the enrichment. This observation may indicate there was indeed an increase in food availability in nutrient-enriched channels, which resulted in lower drift of EPT taxa relative to control channels. Active drift is a known mechanism for patch selection of some EPT taxa with high behavioural drift tendency (Naman et al., 2016). For example, previous experimental manipulations of resource availability have found inverse relationships between food availability and active drift (Hammock and Wetzel, 2013). Furthermore, O’Callaghan et al. (2015) also observed strong nutrient effects on drift patterns, even in the absence of strong effects on invertebrate densities within the experimental units. They attributed this discrepancy to the duration of their experimental manipulations (28 days), arguing their experiment may have been too short to detect long-term changes in community abundance, or shifts in competitive interactions due to changes in food availability (O’Callaghan et al., 2015), a consideration that likely applies to our 22-day manipulation.

Partially in support of our second hypothesis, sediment additions had strong negative effects on benthic invertebrate densities, while also increasing EPT drift flux from our first-order channels. These findings largely support most published literature reporting deleterious effects of sediment on benthic invertebrate communities (e.g., Matthaei et al., 2006; O’Callaghan et al., 2015; Piggott et al., 2015b). Increased inputs of fine sediment have been reported to fill interstitial spaces in stream ecosystems, reducing habitat availability for benthic invertebrates, and causing direct negative effects on sensitive species due to coating of gills and respiratory surfaces (Wood and Armitage, 1997; Allan, 2004; Wagenhoff et al., 2012). Moreover, sediment additions often induce behavioural drift as a response to impaired habitat quality (Connolly and Pearson, 2007; O’Callaghan et al., 2015; Wagenhoff et al., 2012; Piggott et al., 2015b). Specifically, the EPT orders contain several taxa known to respond to sedimentation by drifting short distances in order to find better habitat patches (O’Callaghan et al., 2015; Naman et al., 2016).

On the other hand, our second hypothesis was not supported regarding the effects of sedimentation on leaf decomposition. Instead of reducing leaf processing in the streams, as has been generally reported in the literature (Young et al., 2008;
Tank et al., 2010; Danger et al., 2012; Louhi et al., 2017), sediment additions in isolation actually increased leaf decomposition in first-order channels. This unexpected positive response has been previously reported in a few experimental manipulations of sediment in stream ecosystems (e.g., Matthaei et al., 2010; Piggott et al., 2012, 2015a), and might be due to increased anaerobic respiration of leaf material buried in sediment (Piggott et al., 2015a).

Leaf decomposition was the only response that supported our third hypothesis predicting non-additive interactions among nutrients and sediment in first-order channels. Leaf decomposition showed clear antagonistic effects of the two stressors; while in isolation both stressors tended to increase decomposition, in combination their effect was completely inhibited, resulting in values similar to those of the control treatments. Similar antagonistic nutrient x sediment interactions were reported for measures associated with leaf processing by Piggott et al. (2015a). They observed sediment additions dampened the positive effect of the nutrient enrichment on leaf respiration and cotton tensile strength loss in experimental stream mesocosms. Piggott et al. (2015a) suggested that microbial communities responsible for increased decomposition rates under high nutrient concentrations, had less access to nutrients and oxygen in the water column when sediment was applied (Piggott et al., 2015a). However, in our case the presence of nutrients also dampened the positive effects of sediment, which suggests that nutrient additions inhibited anaerobic microbial respiration in buried leaf packs, a puzzling result that deserves further study.

4.4.2 Tributaries influenced downstream ecosystems through dispersal of sensitive taxa

Partially in agreement with our fourth hypothesis, we detected significant differences between cumulative- and single-stressor tributary treatments. However, to our surprise these differences were observed for variables without significant nutrient x sediment interactions within the tributaries: EPT density and EPT richness. Both EPT metrics were higher in downstream ecosystems with single-stressor tributaries. Because the treatments did not affect EPT density or richness within tribu-
taries, we attribute this result to their opposing effects on EPT drift from tributaries to second-order channels. As mentioned earlier, sediment additions augmented the number of EPT drifting out of first-order channels, nutrient additions decreased it, and the combination of both treatments resulted in additive effects on EPT flux. However, higher EPT density in second-order channels with individual-stressor tributaries seems to suggest that the combination of the two stressors within the same tributary reduced EPT drift further than the addition of the stressors in separate tributaries. The fact that such an interactive effect was not detected in drift responses, may be an artifact of the timing and duration of our first-order drift samples. In an attempt to not disrupt subsidies from tributaries to downstream channels, we collected only two-hour samples during daytime. Previous research suggests that this limited sampling window may not have captured complex interactive effects of the treatments on drift behaviour. For example, in an experimental manipulation of flow carried out at the same experimental facility, Naman et al. (2017) found that drift responses to their treatments were stronger at night-time, indicating that time of the day may impact the likelihood of reliably capturing drift responses to stressors in this particular system.

Our fifth hypothesis, predicting that disturbance in second-order channels would increase proportionally with disturbance in their tributaries, was partially supported for sedimentation rates in second-order channels. However, in contrast to our expectations, most biological responses in second-order channels did not show increasing negative impacts with this trend. On the contrary, the density of EPT taxa, a group generally considered sensitive to organic contamination and habitat degradation, increased proportionally with tributary disturbance. We again attribute this paradoxical result to the impacts of the stressors on the flux of organisms from tributaries to downstream channels. Higher disturbance within the tributaries caused higher EPT drift, likely increasing immigration rates in downstream ecosystems. Even though downstream sedimentation increased with tributary disturbance, there was less sediment deposited per unit area in downstream ecosystems than their first-order tributaries. Thus, individuals emigrating from the tributaries may have found in downstream channels more suitable habitat patches than their source patch. These findings generally fit the mass effects model of the
metacommunity framework. According to this model, even though local habitat conditions and species interactions are important in shaping community composition, high rates of dispersal may override local effects and allow species to persist in unfavourable conditions (Brown et al., 2011). This mass effect has been recognized as a particularly important force in stream networks, where downstream movement of material and individuals from the tributaries may have a disproportionate effect on downstream ecosystems (Campbell Grant et al., 2007).

4.4.3 Implications

We used a simplified model of a stream network to link upstream disturbance with effects on downstream ecosystems. However, we do not contend that a short-term experiment in a small channel network can encompass all processes occurring in real river networks. Our experiment was not realistic in terms of spatial and temporal scales, network complexity, and material inputs from colluvial processes into downstream ecosystems. In spite of these limitations, we argue that some interesting patterns and small-scale processes, such as short-distance dispersal of organisms, dilution, and transfer of materials, could be reliably measured in our experimental channel network. Thus, with some caveats in mind, our study offers interesting insights about the potential impacts of multiple stressor interactions on meta-ecosystem dynamics of river networks.

To our knowledge, this is the first experiment explicitly linking multiple stressor effects in tributaries to effects in downstream recipient ecosystems in a river network. Thus, we had little empirical information to compare our results with. However, our observations generally support previous research highlighting the potential role of spatial species interactions within tributaries on downstream ecosystem function. For instance, Patrick and Fernandez (2013) observed that the spatial distribution of shredder invertebrate species among tributaries, had the potential to regulate particulate organic matter exports from headwaters to downstream ecosystems, due to competitive interactions.

Our results showed that stressor additions in tributaries can strongly influence
ecological function and structure of downstream ecosystems. In our experimental system, most of these effects were due to impacts on dispersal patterns of sensitive taxa, underscoring the importance of metacommunity frameworks to understand how disturbances at the scale of the tributaries may influence population dynamics in downstream ecosystems (Brown et al., 2011; Campbell Grant et al., 2007).

Additionally, our observations in first-order channels further support previous studies reporting that deposited fine sediment is a predominant determinant of invertebrate community dynamics and ecological functioning in stream ecosystems (Louhi et al., 2017; Piggott et al., 2015b). As such, our results suggest that management measures that reduce the input of fine sediment into headwater streams should be prioritized to restore functional and structural integrity of stream ecosystems.
Table 4.1: ANOVA summary of linear models evaluating impacts of the stressor treatments on response variables of the first-order channels. Significant effects ($P < 0.05$) are indicated in bold.

<table>
<thead>
<tr>
<th>Response variables</th>
<th>df</th>
<th>N</th>
<th>S</th>
<th>$N*S$</th>
<th>F</th>
<th>P</th>
<th>F</th>
<th>P</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Habitat variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sediment dry mass</td>
<td>1,21</td>
<td>0.592</td>
<td>0.450</td>
<td>114.206</td>
<td>$&lt;0.0001$</td>
<td>1.061</td>
<td>0.315</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sediment AFDM</td>
<td>1,21</td>
<td>2.218</td>
<td>0.151</td>
<td>11.349</td>
<td>0.003</td>
<td>2.284</td>
<td>0.146</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sediment % organic</td>
<td>1,21</td>
<td>0.984</td>
<td>0.333</td>
<td>19.115</td>
<td>$&lt;0.0001$</td>
<td>0.823</td>
<td>0.375</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Benthic invertebrates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total density</td>
<td>1,18</td>
<td>0.002</td>
<td>0.966</td>
<td>5.975</td>
<td><strong>0.025</strong></td>
<td>0.000</td>
<td>0.988</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total richness</td>
<td>1,18</td>
<td>0.240</td>
<td>0.630</td>
<td>0.004</td>
<td>0.953</td>
<td>2.452</td>
<td>0.135</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPT density</td>
<td>1,18</td>
<td>0.320</td>
<td>0.578</td>
<td>1.343</td>
<td>0.262</td>
<td>0.576</td>
<td>0.458</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPT richness</td>
<td>1,18</td>
<td>0.110</td>
<td>0.744</td>
<td>0.504</td>
<td>0.487</td>
<td>2.440</td>
<td>0.136</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scraper density</td>
<td>1,18</td>
<td>1.483</td>
<td>0.239</td>
<td>0.007</td>
<td>0.934</td>
<td>1.144</td>
<td>0.299</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shredder density</td>
<td>1,18</td>
<td>0.298</td>
<td>0.592</td>
<td>1.270</td>
<td>0.275</td>
<td>0.381</td>
<td>0.545</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Predator density</strong></td>
<td>1,18</td>
<td>0.062</td>
<td>0.806</td>
<td>10.492</td>
<td><strong>0.005</strong></td>
<td>0.016</td>
<td>0.902</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collector density</td>
<td>1,18</td>
<td>0.035</td>
<td>0.854</td>
<td>3.585</td>
<td>0.074</td>
<td>0.065</td>
<td>0.801</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>drift rate day 2</td>
<td>1,18</td>
<td>0.033</td>
<td>0.857</td>
<td>0.828</td>
<td>0.374</td>
<td>0.132</td>
<td>0.720</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>drift rate day 22</td>
<td>1,18</td>
<td>1.769</td>
<td>0.205</td>
<td>0.087</td>
<td>0.773</td>
<td>0.023</td>
<td>0.881</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ecosystem function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf decomposition</td>
<td>1,18</td>
<td>2.351</td>
<td>0.141</td>
<td>2.185</td>
<td>0.155</td>
<td>8.520</td>
<td><strong>0.008</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Table 4.2: Parameter estimates of the zero-inflated Poisson regression explaining EPT drift flux from first-order channels on day 22. The model includes two components: a count model (Poisson with log link), and a zero-inflation model (binomial with logit link). Significant effects ($P < 0.05$) are indicated in bold.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Estimate</th>
<th>SE</th>
<th>z</th>
<th>$P$</th>
<th>Estimate</th>
<th>SE</th>
<th>z</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1.735</td>
<td>0.307</td>
<td>5.650</td>
<td>&lt; 0.0001</td>
<td>3.302</td>
<td>154.596</td>
<td>0.021</td>
<td>0.983</td>
</tr>
<tr>
<td>S</td>
<td>-0.619</td>
<td>0.307</td>
<td>-2.060</td>
<td>0.044</td>
<td>-2.689</td>
<td>154.596</td>
<td>-0.017</td>
<td>0.986</td>
</tr>
<tr>
<td>N*S</td>
<td>-0.015</td>
<td>0.307</td>
<td>-0.034</td>
<td>0.973</td>
<td>2.969</td>
<td>154.596</td>
<td>0.019</td>
<td>0.985</td>
</tr>
</tbody>
</table>
Table 4.3: Summary of independent *t*-test’s to compare response variables in second-order channels fed by tributaries where the nutrients and sediment were applied in combination (cumulative; tributary treatment b in Figure 4.2) and second-order channels fed by tributaries where the stressors were applied separately (single; tributary treatment c in Figure 4.2). Significant effects (*P* < 0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Cumulative</th>
<th>Single</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Habitat variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sediment dry mass (g m^-2)</td>
<td>133.53</td>
<td>113.53</td>
<td>502.79</td>
<td>249.26</td>
</tr>
<tr>
<td>Sediment AFDM (g m^-2)</td>
<td>16.18</td>
<td>3.68</td>
<td>23.82</td>
<td>15.29</td>
</tr>
<tr>
<td>Nitrate (µg L^-1)</td>
<td>76.5</td>
<td>0.5</td>
<td>80.5</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Benthic invertebrates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total density (ind m^-2)</td>
<td>1083.3</td>
<td>770.1</td>
<td>823.6</td>
<td>420.3</td>
</tr>
<tr>
<td>Total richness (taxa per mesocosm)</td>
<td>11.3</td>
<td>3.1</td>
<td>11.7</td>
<td>2.3</td>
</tr>
<tr>
<td>EPT density (ind m^-2)</td>
<td>156.9</td>
<td>44.9</td>
<td>254.9</td>
<td>37.0</td>
</tr>
<tr>
<td>EPT richness (taxa per mesocosm)</td>
<td>3.3</td>
<td>0.6</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Scraper density (ind m^-2)</td>
<td>44.1</td>
<td>29.4</td>
<td>122.5</td>
<td>44.9</td>
</tr>
<tr>
<td>Shredder density (ind m^-2)</td>
<td>39.2</td>
<td>30.6</td>
<td>58.8</td>
<td>58.8</td>
</tr>
<tr>
<td>Predator density (ind m^-2)</td>
<td>78.4</td>
<td>55.7</td>
<td>39.2</td>
<td>22.5</td>
</tr>
<tr>
<td>Collector density (ind m^-2)</td>
<td>142.1</td>
<td>51.6</td>
<td>122.5</td>
<td>17.0</td>
</tr>
<tr>
<td>drift rate day 2 (ind h^-1)</td>
<td>1.3</td>
<td>1.2</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>drift rate day 22 (ind h^-1)</td>
<td>0.3</td>
<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Ecosystem function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf decomposition (g AFDM lost)</td>
<td>0.5</td>
<td>0.1</td>
<td>0.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>
**Table 4.4:** ANOVA summary of linear models evaluating the impact of disturbance level within the tributaries on response variables of second-order channels. Significant effects ($P < 0.05$) are indicated in bold.

<table>
<thead>
<tr>
<th>Stress level</th>
<th>Response variables</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Habitat variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sediment dry mass</td>
<td>2,6</td>
<td>19.024</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Sediment AFDM</td>
<td>2,6</td>
<td>13.852</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Sediment % organic</td>
<td>2,6</td>
<td>2.113</td>
<td>0.202</td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>2,6</td>
<td>0.106</td>
<td>0.901</td>
<td></td>
</tr>
<tr>
<td><strong>Benthic invertebrates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total density</td>
<td>2,6</td>
<td>1.344</td>
<td>0.329</td>
<td></td>
</tr>
<tr>
<td>Total richness</td>
<td>2,6</td>
<td>4.167</td>
<td>0.081</td>
<td></td>
</tr>
<tr>
<td><strong>EPT density</strong></td>
<td>2,6</td>
<td>5.187</td>
<td><strong>0.049</strong></td>
<td></td>
</tr>
<tr>
<td>EPT richness</td>
<td>2,6</td>
<td>1.716</td>
<td>0.232</td>
<td></td>
</tr>
<tr>
<td>Scraper density</td>
<td>2,6</td>
<td>1.972</td>
<td>0.220</td>
<td></td>
</tr>
<tr>
<td>Shredder density</td>
<td>2,6</td>
<td>2.197</td>
<td>0.192</td>
<td></td>
</tr>
<tr>
<td>Predator density</td>
<td>2,6</td>
<td>1.839</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>Collector density</td>
<td>2,6</td>
<td>0.736</td>
<td>0.518</td>
<td></td>
</tr>
<tr>
<td>drift rate day 2</td>
<td>2,5</td>
<td>0.299</td>
<td>0.754</td>
<td></td>
</tr>
<tr>
<td>drift rate day 22</td>
<td>2,6</td>
<td>1.760</td>
<td>0.264</td>
<td></td>
</tr>
<tr>
<td><strong>Ecosystem function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf decomposition</td>
<td>2,6</td>
<td>0.300</td>
<td>0.751</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1: Photo of the experimental channel-network set-up consisting in 24 first-order channels converging in pairs to feed 12 second-order channels (a). Detail of a pair of first-order channels and their second-order recipient (b).
Figure 4.2: Schematic representation of our experimental channel-network set up. Each pair of first-order channels (represented as gray bars) is a treatment for its second-order receptor (black bars). Initials are used to represent stressor additions in first-order treatments (N, nutrients; S, sediment; N+S, nutrient + sediment). Letters are used to represent second-order tributary treatments (a, two control tributaries; b, one control tributary and one tributary with both stressors; c, nutrients added in one tributary and sediment in the other; and d, nutrients and sediment added in both tributaries). There were three replicates of each tributary treatment for a total of 12 second-order channels and 24 first-order channels.
Figure 4.3: Standardized effect size (Hedge’s $d \pm 95\%$ CI) for significant main and interactive effects of the stressors on first-order channels. Letters are used to represent main effects (N, nutrients; S, sediment) and interactions (N*S, nutrients x sediment). For main effects positive values indicate increases in the response variable, whereas negative values indicate the opposite. For interactions confidence intervals overlapping zero indicate additive interactions, positive values denote synergies, and negative values indicate antagonisms. Symbols are used to represent significance according to the linear models presented in Table 4.1: $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, $P < 0.1$. 
Figure 4.4: Bar plot illustrating significant antagonistic effects of the stressors on leaf decomposition (as AFDM loss from 3 g leaf packs). Bars represent the mean of the treatments (± SE): control (n = 9), sediment (S, n = 3), nutrients (N, n = 3), nutrients + sediment (N+S, n = 9). A dotted line represents the predicted additive effect of the two stressors. Letters indicate significant differences among the treatments according to Tukey’s Honestly Significant Difference tests.

Figure 4.5: Plots representing the impact of the stressor treatments on the frequency of zeros (a) and log-transformed counts (b) of individuals from the orders Ephemeroptera, Plecoptera, and Trichoptera drifting out of the first-order channels on day 22. Letter notation for the treatments is consistent with Figure 4.4.
Figure 4.6: Bar plots illustrating the effect of tributary level of disturbance on total sediment deposition (a), organic sediment deposition (b), and EPT density (c) in recipient second-order channels. Treatments in the x-axis represent: no disturbance in the tributaries (0, tributary treatment a in Figure 4.2), one tributary disturbed (1, tributary treatment b in Figure 4.2), two tributaries disturbed (2, tributary treatment d in Figure 4.2). Bars represent the mean of each tributary treatment (±SE, n = 4). Letters indicate significant differences among the treatments according to Tukey’s Honestly Significant Difference tests.
Chapter 5

Conclusions: synthesis and implications

5.1 Overview

My thesis presented a detailed, mechanistic investigation of freshwater ecosystem responses to three widespread agricultural stressors, i.e. nutrient enrichment, sedimentation, and insecticide contamination. These studies constitute some of the first experimental evaluations of three-way interactions among these stressors, and addressed two freshwater ecosystem types, encompassing processes occurring at the scale of communities, ecosystems, and meta-ecosystems (river networks). My results contribute novel evidence suggesting that nutrient enrichment, sedimentation, and insecticide contamination often interact non-additively, as I observed multiple antagonistic and reversal interactions altering functioning and biological composition of lentic and lotic ecosystems at different scales of organization.

In my first experiment (Chapter 2), I observed that even low concentrations of the insecticide chlorpyrifos have the potential to interact with fine sediment, exerting deleterious impacts on small-sized invertebrates in a closed stream community. Furthermore, my results suggested that even though chlorpyrifos did not strongly
reduce invertebrate density, it still altered organic matter processing by shredding invertebrates, which resulted in significant reductions in leaf decomposition rates.

With my second experiment (Chapter 3), I demonstrated that open, lentic ecosystems are also susceptible to complex interactions among nutrients, sediment, and insecticide contamination, as I observed that imidacloprid frequently interacted antagonistically with moderate levels of nutrient enrichment and sedimentation. Moreover, my results indicated that imidacloprid has the potential to influence ecosystem metabolism through impacts on primary consumers.

Finally, in my third experiment, I explored processes occurring at the scale of the river network meta-ecosystem, and observed that complex multiple-stressor interactions have the potential to strongly alter the flux of organisms from tributaries to downstream ecosystems. Furthermore, my results suggested that, at small spatial and relatively short time scales, these alterations of within-network migration patterns, may be more influential than the transmission of disturbances (e.g., nutrient and sediment loads) from headwaters to downstream recipient ecosystems.

5.2 Single- and multiple-stressor responses across three freshwater mesocosm manipulations

In this section I discuss response patterns observed across the three experimental manipulations described in this dissertation. Even though the response variables analyzed here are not strictly independent, they show trends that were consistent across two different ecosystem types and three different scales of biological organization.

5.2.1 Single stressor effects: the disproportionate impacts of sedimentation

Across the three experiments conducted for this dissertation, I evaluated 51 univariate biological responses for both nutrient enrichment and sediment, 20 responses for chlorpyrifos, and 19 responses for imidacloprid (Figure 5.1a). In both lentic and
lotic ecosystems, and across all scales measured in these experiments, sedimentation was consistently the most detrimental of the three types of agricultural stressors evaluated. Sediment additions significantly affected 17 biological responses, with negative effects on 15 variables and positive effects on only 2 (leaf decomposition, EPT drift flux, Figure 5.1a). These observations agree with literature suggesting that increased fine sediment inputs in agricultural landscapes are among the most pervasive ecological stressors for stream (Matthaei et al., 2010; Piggott et al., 2015b; Louhi et al., 2017) and lentic ecosystems (Luo et al., 1997; Skagen et al., 2008; Wood and Richardson, 2009). Furthermore, the observed impact mechanisms generally support previous experimental evidence. In keeping with a number of previous investigations (e.g., Bilotta and Brazier, 2008; Wood and Richardson, 2009; Wagenhoff et al., 2012; Piggott et al., 2015b; Louhi et al., 2017), I observed that fine sediment additions consistently reduced benthic and pelagic invertebrate density, mostly due to physical impacts on sensitive taxa (e.g., Ephemeroptera, Plecoptera, and Trichoptera), and bottom-up effects from reduced primary production and impaired leaf decomposition. However, my studies also offered new insights about the mechanisms by which the impacts of sedimentation scale up from communities to ecosystems and meta-ecosystems. With the pond mesocosm manipulation, I was able to establish clear cause-effect associations between inorganic sediment inputs and strong alterations of ecosystem metabolism. Moreover, with the experimental channel network, I demonstrated that sedimentation in tributary channels had unexpected effects on species composition of downstream ecosystems, as it promoted migration of sensitive invertebrate species within the river network meta-ecosystem.

The second most influential stressor was nutrient enrichment, which had positive effects on 5 (9.8%) of the variables tested, and negative effects on only one variable (EPT drift flux, Figure 5.1a). This was not surprising because moderate nutrient additions have been extensively documented to produce positive bottom-up effects on benthic and pelagic food webs in oligotrophic freshwater ecosystems (Lienesch et al., 2005; Shurin et al., 2012). However, all these significant effects were observed in pond mesocosms. In contrast to many investigations finding strong effects of nutrient enrichment in stream mesocosm experiments (Piggott
et al., 2015b; García et al., 2017), the application of nutrients did not cause strong responses in either of my stream experiments. This unexpected result indicates that nutrient additions in these experiments were not sufficient to cause a strong response that would be detectable by the time of sampling. However, in both stream experiments I detected patterns that suggest there was an increase in biomass of primary producers that was quickly transferred to higher trophic levels (Wagenhoff et al., 2012). For instance, in the microcosm experiment nutrient tanks had on average higher periphyton biomass and higher invertebrate biomass, but these responses were not statistically significant due to their large variation among enriched tanks. In the channel network experiment, I did not measure periphyton but there was lower emigration of Ephemeroptera, Plecoptera, and Trichoptera taxa from enriched channels, suggesting higher food availability due to the enrichment (Hammock and Wetzel, 2013).

The results of this investigation also highlighted the influential role of insecticide contamination in lentic and lotic ecosystems, contributing new evidence of the potential indirect impacts of insecticides on freshwater ecosystem function. Even at relatively low concentrations, I observed significant negative effects of insecticide contamination on 2 (4%) of the biological responses tested (Figure 5.1a). This is a considerable proportion, taking into account these were aggregated community and ecosystem responses, and do not measure taxon-specific effects of the insecticides. Out of these significant responses, one was due to direct toxic effects of imidacloprid on zooplankton communities, and the remaining was documented for indirect effects of chlorpyrifos on ecosystem function. Some of these responses, such as the negative effects of chlorpyrifos on leaf decomposition, had been previously documented in experimental and field studies (Brock et al., 1992a; Cuppen et al., 1995; Van den Brink et al., 1996). However, this is one of the first studies documenting significant negative impacts of imidacloprid on zooplankton density at concentrations that are not considered harmful according to standardized toxicity tests (Sánchez-Bayo and Goka, 2006). Moreover, I observed marginally significant effects of imidacloprid on net ecosystem metabolism, a potential indirect effect that has not been previously reported in the literature. These findings contribute to existing evidence suggesting that impacts of neonicotinoid insecticides may be
stronger in realistic scenarios than predicted by acute toxicity tests (Sánchez-Bayo et al., 2016).

5.2.2 Cumulative effects: the importance of antagonistic and reversal interactions

Out of the 168 stressor combinations evaluated across the three studies, 157 (94%) were additive and 11 (6%) resulted in significant interactive effects (Figure 5.1b). From these non-additive interactions, 9 (82%) were antagonistic and 2 (8%) were reversals. The importance of antagonistic interactions on freshwater ecosystems has been previously underscored by Jackson et al. (2016), who observed that antagonisms comprised 41% of the 286 stressor combinations evaluated in their meta-analysis. However, my results differed from the trend documented by Jackson et al. (2016), who observed that antagonistic interactions occurred most frequently on functional metrics, while diversity metrics showed mostly additive responses. In my studies, most antagonisms were observed for diversity metrics, while functional metrics exhibited mostly additive responses to cumulative stressors. This discrepancy further reinforces the notion that the outcome of multiple-stressor interactions is strongly determined by the context and the specific characteristics of the receptor biological systems (Segner et al., 2014; Clements et al., 2016).

The importance of context-dependent responses to multiple stressors has been recently highlighted by Clements et al. (2016), who observed that our ability to predict variation in the response to stressors among communities is still limited. My studies contribute new evidence to this research gap, demonstrating that characteristics at the scale of microhabitats may have a disproportionate influence on the response of aquatic invertebrates to cumulative stressors. My finding of antagonistic effects on species diversity also supports the notion of context-dependency, as it suggests that community composition and tolerance patterns also determine responses to stressors. Antagonistic impacts on diversity suggest that, in my focal communities, species tolerances to the stressors were positively correlated. Hence, each stressor eliminated certain species but was likely to leave species that were tolerant to the other stressors, a pattern that has been denominated “stress-induced
community tolerance” (Vinebrooke et al., 2004). Most importantly, the prevalence of additive effects on functional measures of these systems indicates that the remaining species were not able to compensate functionally for the species loss (Jackson et al., 2016), offering new evidence of potential community and ecosystem-level consequences of the development of community tolerance in freshwater ecosystems.

Although I did not observe synergistic interactions according to the classification framework I used, one of the reversal interactions I documented ultimately resulted in effects that were more detrimental than predicted by additivity. This is an interesting observation that further reinforces the importance of considering reversal interactions in multiple-stressor classification frameworks (Piggott et al., 2015c; Jackson et al., 2016). With additive classification frameworks that do not account for reversals, (e.g., the classification framework used by Crain et al., 2008), the above interaction would have been classified as an antagonism, which would have clearly underestimated its effects on freshwater communities. Reversal interactions are important because they represent the most extreme cases of “ecological surprises”, where the cumulative impact of two stressors actually goes in the opposite direction as predicted by their single effects (Piggott et al., 2015c; Jackson et al., 2016). According to my results they should be carefully considered in multiple-stressor studies, because they could represent instances where the presence of one stressor inverts the effect of the dominant stressor with potential deleterious effects on freshwater ecosystems (Jackson et al., 2016).

In agreement with my initial expectations, sediment x insecticide was the stressor combination that most frequently showed complex interactions (Figure 5.1b). The potential negative effects of pesticide-contaminated sediments on freshwater ecosystems have been recognized for some time (Warren et al., 2003; Burton and Johnston, 2010). Because some insecticides are moderately hydrophobic, they tend to adsorb onto sediment particles when they enter the water column (Brock et al., 1992a; Pablo et al., 2008; Gebremariam, 2011). Thus, deposited bed sediments may become a sink for insecticides, thereby increasing the exposure of benthic organisms through contact or ingestion of contaminated particles (Warren et al., 2003; Burton and Johnston, 2010). However, my studies are some of the first at-
tempts to explicitly evaluate potential synergistic interactions among sedimentation and insecticides at the scale of communities and ecosystems. As such, my results provide novel evidence that increased fine sediment deposition has the potential to enhance the toxic effects of insecticides like chlorpyrifos on benthic invertebrate communities.

Interestingly, the presence of fine sediment did not consistently enhance the negative effects of the insecticides in my experiments (Figure 5.2). Sediment additions actually seemed to mask the negative effects of imidacloprid on pelagic invertebrate communities in the pond mesocosms. Because this was the first experiment evaluating multiple stressor interactions between imidacloprid and sedimentation, I did not have empirical information with which to contrast my results. However, according to my observations, the presence of both stressors in the system caused the stress-induced community tolerance pattern described in previous paragraphs (Vinebrooke et al., 2004). A similar response was observed for the combination of nutrients and the two insecticides (Figure 5.2), all of which were antagonistic and affected diversity variables, supporting results of previous empirical evaluations (Alexander et al., 2013, 2016). Finally, in my studies, nutrients and fine sediment only interacted non-additively once, which contrasts with previous examinations that have found frequent complex interactions for this stressor combination (e.g., Townsend et al., 2008; Piggott et al., 2012, 2015b). This is not entirely surprising because these complex interactions have been reported for stream ecosystems and my nutrient additions did not exert strong impacts in either of my stream mesocosm experiments.

## 5.3 Implications

My studies contribute new evidence suggesting that complex interactions among nutrient enrichment, sedimentation, and insecticide contamination are common in different types of freshwater ecosystems, and have distinct mechanisms operating at different scales of organization. As such, my findings support a growing body of literature underscoring the need to account for potential detrimental effects arising from these interactions, in order to control and mitigate agricultural impacts on
freshwater biodiversity and function (Matthaei et al., 2010; Piggott et al., 2015b; Liess et al., 2016).

My results generally support the notion that in order to predict the outcome of multiple-stressor interactions it is necessary to shift from focusing solely on stressor characteristics, to focusing also on the properties of the receptor systems (Rohr et al., 2006; Segner et al., 2014). According to my observations, physical and chemical habitat characteristics, the degree of connectivity of the ecosystem, and the patterns of species co-tolerance to the individual stressors, are major determinants of the outcome of multiple-stressor interactions. An important consequence of this context-dependency is that even though most significant interactions observed in these experiments were antagonistic, in a different context (e.g. different microhabitat characteristics or different stressor levels), similar stressor combinations could result in synergistic effects or reversals, with potentially deleterious consequences for freshwater ecosystems. Moreover, these observations suggest that theoretical frameworks like the community co-tolerance hypothesis (Vinebrooke et al., 2004) may be good starting points to predict cumulative stressor effects on biological diversity. However, many more empirical studies are needed to better understand how multiple stressors impact diversity, and how these impacts on diversity will ultimately translate into impacts on freshwater ecosystem functioning (Sandin and Solimini, 2009). For instance, such studies could involve the manipulation of species assemblages to evaluate multiple-stressor effects on communities that are predicted to exhibit stress-induced tolerance.

One of the most important findings of this investigation is that environmentally relevant levels of insecticide contamination may interact non-additively with nutrient enrichment and sedimentation, and cause important effects on ecosystem function. For example, I found that the presence of fine sediment in the substrate has the potential to enhance insecticide toxicity to small invertebrates. This observation has major implications given the widespread co-occurrence of these two stressors in freshwater ecosystems around the world (Vörösmarty et al., 2010; Schäfer et al., 2016), and highlight the importance of including evaluations of sediment-bound toxicity in risk assessment protocols for pesticides in freshwater ecosystems. Furthermore, across my studies sublethal insecticide exposures consistently altered
ecosystem processes through effects on trophic interactions. These findings suggest that risk assessment frameworks based on single-species toxicity tests may underestimate impacts on freshwater ecosystem function, as they cannot account for indirect effects through disruption of species interactions or complex interactions with other agricultural stressors. In this sense, my results support a growing body of literature showing that mesocosm experiments are a valuable complementary tool for pesticide risk assessment, which allows controlled and statistically robust evaluations of toxicant effects in more realistic scenarios (Culp et al., 2000; Clements, 2004; Alexander et al., 2016).

Finally, my experiments at different scales of ecological organization consistently showed that the effects at higher levels of organization cannot be easily predicted from impacts detected at smaller, nested scales (e.g., impacts at the meta-ecosystem scale are not easy to predict from impacts on local ecosystems). For instance, I detected strong negative effects of sediment inputs on net ecosystem productivity of the pond mesocosms, despite the fact that there were not significant periphyton or phytoplankton biomass responses when analyzed separately. Another example of surprising emergent properties in my study systems were the unexpected positive impacts of headwater disturbances on downstream ecosystems, due to mass effects within the river network meta-ecosystem. Together, these findings highlight the importance of choosing the correct scale of measurement to assess ecological condition, and underscore the usefulness of whole-ecosystem experiments to reliably evaluate stressor impacts on ecosystem structure and function (Loreau et al., 2003; Buck et al., 2004).

The key management implication of my results is that sedimentation, nutrient enrichment, and insecticide pollution should be managed together in order to effectively protect freshwater ecosystems in agricultural landscapes. Freshwater management decisions should take into account that, even at relatively low levels, these stressors may act in combination to cause unexpected negative effects on aquatic biota and ecosystem processes. In particular, my results suggest that sedimentation was the most detrimental stressor and had the potential to enhance toxicity of hydrophobic insecticides. Thus, management strategies that reduce the amount of fine sediment entering aquatic ecosystems, such as soil conservation practices and
stabilization structures, should be a priority for restoration efforts. Additionally, the establishment of riparian vegetation buffers may be a very effective measure to mitigate the impacts of agriculture, as they can control nutrients, pesticides, and fine sediment entering water bodies via runoff (Udawatta et al., 2008; Lin et al., 2011; Chará et al., 2011).
Figure 5.1: Frequency distribution of individual stressor effects (a), and multiple-stressor interactions (b) on freshwater ecosystem responses evaluated across the three experiments conducted for this dissertation. Letters are used to represent individual stressor treatments (N, nutrients; S, sediment; I, insecticide) and interactions (N*S, nutrient x sediment; N*I, nutrient x insecticide; S*I, sediment x insecticide; N*S*I; nutrient x sediment x insecticide). The number of response variables analyzed for each stressor (a) or stressor combination (b) is indicated in parentheses.
Figure 5.2: Distribution of significant multiple-stressor interactions involving insecticides across the experiments. Letters are used to denote the treatments: nutrients (N), sediment (S), imidacloprid (IMI), and chlorpyrifos (CPY).
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Appendix A

Supporting Materials
Table A.1: ANOVA summary of linear mixed effects models to test impacts of stressor treatments (fixed effects) and time (week: fixed effect) on dissolved oxygen, temperature, pH, and conductivity, with individual microcosm treated as a random effect. Symbols are used to represent significance of the effects according to the ANOVAs: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, $P < 0.1$.

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Figure A.1: Schematic of the stream microcosm set-up. Darker rectangle in the middle of the tank represents a horizontal glass plate introduced to partially divide the tank into an upper and a lower section. A bubbler along with a plastic deflector placed in one end of the tank promoted circular flow in the direction indicated by the blue arrows. Redrawn from Sanpera-Calbet et al. (2012).
Figure A.2: Untransformed univariate responses of gravel invertebrates across the treatments on day 15. Boxes are drawn around upper and lower quartiles with whiskers indicating maximum and minimum values, dark thick lines indicate the median, and points denote outliers. Y-axis does not always start in zero.
Figure A.3: Untransformed univariate responses of leaf invertebrates across the treatments on day 15. Boxes are drawn around upper and lower quartiles with whiskers indicating maximum and minimum values, dark thick lines indicate the median, and points denote outliers. Y-axis does not always start in zero.
Figure A.4: Untransformed ecosystem processes across the treatments on day 15. Boxes are drawn around upper and lower quartiles with whiskers indicating maximum and minimum values, dark thick lines indicate the median, and points denote outliers. Y-axis does not always start in zero.