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Testing the effects of food quality, as well as chemical contamination of water and sediments, on aquatic detrital systems using laboratory experiments

A thesis submitted by Libe Solagaistua Zabala to the University of the Basque Country for the degree of Doctor of Philosophy, under the supervision of Dr. Aitor Larrañaga Arrizabalaga.

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eman ta zabal zazu
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Laburpena

Materia organikoaren kalitate aldaketek eta kutsadura kimikoak sistema detritikoetan duten eragina aztertzea izan da tesi honen helburu nagusia. Inpaktu desberdinek duten eragina aztertzeko, mikrokosmo esperimentuak erabili dira tesian zehar. Errekara iristen den materia organikoaren kalitatea eta kalitate altuko materialen eskuragarritasuna inguruko lur erabileren araberakoak dira. Lehenengo kapituluan materia organikoaren kalitateak anfipodoen kontsumoan eta portaeran duten eragina aztertu genuen. Materia organikoaren kalitateak esangarriki aldatu zuen *Echinogammarus berilloni* zatitzailearen kontsumoa baina kalitate altueneko materiala ez zen kontsumituena izan. Kontsumitutako materia organikoaren kalitateak ez zuen indibiduoen portaera (RNA:BM, hazkuntza, gorputz egoera eta lipido kantitatea) esangarriki aldatu, nahiz eta gorputz egoerak esangarriki erantzun zuen. Esperotakoaren kontrara, kontsumo tasa eta RNA:BM ratioa ezin dira seinale goiztiar bezala erabili. Inkubazio denborak inkoherentziak sortzen ditu neurtutako aldagaietan eta gure emaitzak kontutan hartuta, esperimentu mota hauetan 16 eguneko inkubazioa minimoa gomendatzen dugu. Bigarren kapituluan ikusi genuen kalitate altuko materia organikoaren eskuragarritasunak kontsumo tasak estimulatzen zituela baina ez zuen eragin nabarmenik izan zatitzaileen portaeran. Araztegitik eratorritako efluenteak komunitate mikrobiarraren eta zatitzaileen portaeran eta materia organikoaren kontsumoan duen eragina aztertu zen hirugarren kapituluan. Efluentearen eragina nabarmenagoa izan zen laborategiko esperimentuan mendiko esperimentuan baino, baliteke efluentearen ibaiko diluzio handiaren eraginez. Laborategiko esperimentuan (efluente kontzentrazioa 0 %tik 100 %era), efluenteak orokorrean komunitate mikrobiarraren eta zatitzaileen portaera aktibatu zuen. Laugarren kapituluan, sedimentuetako kutsatzaile nahasketa baten eragina aztertu genuen *Monoporeia affinis* indibiduoetan biomarkatzaile desberdinak neurtuta. Bi esposizio egoera sortu genituen: esposizio kronikoa kutsadura maila baxuarekin eta kontzentrazio altuko esposizio laburrak. Kontzentrazio-denbora balio altueneko tratamenduak eragin nabarmenagoak izan zituen indibiduoengan. Kutsadura gradienteari linealki eta ez-linealki erantzuten dioten biomarkatzaileen arteko korrelazioak indibiduoek jasotzen duten estres maila detektatzeko tresna egokia zirela ikusi genuen.

Summary

The general objective of this thesis was to assess the effect of food quality and chemical contamination on detrital systems. In order to test the effects of the different drivers a model ecosystem microcosm experiment approach was used throughout the thesis. In the first chapter the effect of organic matter quality and the availability of high quality leaf litter, which can be altered by land use changes, were tested on amphipod consumption rate and performance. Shredder's (*Echinogammarus berilloni*) consumption rate was significantly affected by organic matter quality but the highest quality leaf species was not the most consumed one. Detritivore performance (RNA:BM, growth rate, mass body condition and lipid body condition) was not strongly affected by consumed organic matter quality, although mass body condition showed a significant response. Contrary to expectations, our study did not support the use of consumption rate and RNA:BM ratio as early warning signals. Incubation time creates inconsistencies in the measured responses and based on our results, we recommend a minimum of 16 days of incubation for this type of experiments. In the second chapter we observed that supplementing the predominant organic matter with high quality resource stimulated the consumption rates of low quality resource, whereas amphipod performance manifested subtle responses. In the third chapter, the effect of a wastewater treatment plant (WWTP) effluent was assessed on microbial and detritivore performance, and on organic matter processing. Response to WWTP effluent was clearer in the laboratory than in the field experiment, probably due to the high dilution of the effluent in stream water. In the laboratory, were the effluent concentration ranged from 0 % to 100 %, a general subsidy response (microbial and detritivore performance) was observed. In the fourth chapter the effect of a mixture of chemicals in the sediments was determined by means of biomarkers measured on the amphipod *Monoporeia affinis*. Two different exposure scenarios were created: chronic exposure to a low dose of pollutants and acute exposure to a high dose of pollutants. The treatment with the largest concentration-time product (acute exposure) was the most detrimental for the individuals. Correlations between biomarkers that respond linearly (monotonically) and non-linearly to the pollution gradient demonstrated to be a useful tool to determine the stress level the consumer were subjected to.

Resumen

El objetivo general de esta tesis fue evaluar el efecto de la calidad de la hojarasca y la contaminación química en los sistemas detríticos. A fin de probar los efectos de los diferentes impulsores, se utilizó un enfoque experimental de ecosistemas modelo en microcosmos. En el primer capítulo, se evaluó el efecto de la calidad de la materia orgánica y la disponibilidad de hojarasca de alta calidad, que pueden ser alterados por los cambios en el uso del suelo, en la tasa de consumo y el rendimiento de los anfípodos. La tasa de consumo de los fragmentadores (*Echinogammarus berilloni*) se vio significativamente afectada por la calidad de la materia orgánica, pero la hojarasca de mayor calidad no fue la más consumida. El rendimiento de los detritívoros (ARN:BM, tasa de crecimiento, condición corporal y condición corporal lipídica) no se vio muy afectado por la calidad de la materia orgánica consumida, aunque la condición corporal mostró una respuesta significativa. Al contrario de lo esperado, nuestro estudio no apoyó el uso de la tasa de consumo y la relación ARN:BM como señales de alerta temprana. El tiempo de incubación crea inconsistencias en las respuestas medidas y, según nuestros resultados, recomendamos un mínimo de 16 días de incubación para este tipo de experimentos. En el segundo capítulo observamos que complementar la materia orgánica predominante con recursos de alta calidad estimuló las tasas de consumo de recursos de baja calidad, mientras que el rendimiento de los anfípodos manifestó respuestas sutiles. En el tercer capítulo, se evaluó el efecto de un efluente de una planta de tratamiento de aguas residuales (EDAR) en el rendimiento microbiano y detritívoro, y en el procesamiento de materia orgánica. La respuesta al efluente de la EDAR fue más clara en el laboratorio que en el experimento de campo, probablemente debido a la alta dilución del efluente en el río. En el laboratorio, donde la concentración del efluente varió de 0 % a 100 %, se observó una respuesta general de subsidio (rendimiento microbiano y detritívoro). En el cuarto capítulo, se determinó el efecto de una mezcla de sustancias químicas en los sedimentos por medio de biomarcadores medidos en el anfípodo *Monoporeia affinis*. Se crearon dos escenarios de exposición: exposición crónica a una baja dosis de contaminantes y exposición aguda a una alta dosis de contaminantes. El tratamiento con el mayor valor de concentración-tiempo (exposición aguda) fue el más perjudicial para los individuos. Las correlaciones entre biomarcadores que responden de forma lineal (monotónica) y no lineal al gradiente de contaminación demostraron ser una herramienta útil para determinar el nivel de estrés al que los individuos son sometidos.

General introduction

General introduction

Microcosm experiments are useful tools…

Global environmental change is driven by human activity (Rockström *et al.*, 2009). Understanding the present and future effects of those changes on the structure and functioning of ecosystems is one of the main concerns of ecologists. In this sense, researchers are using different approaches to identify and predict ecosystem-level responses to different impacts. On the one hand, field experiments, both observational and manipulative (e.g., Arroita et al., 2016; Ponsatí et al., 2016), offer direct valuable information of the tested effects as the responses are measured *in situ* at the ecosystem level. However, in these experiments, there are nearly always confounding factors that challenge isolating the effect of the tested impact from other environmental variables that could also affect the response. Additionally, the comparability between field experiments is very limited by the idiosyncratic nature of the selected study sites. On the other hand, we have standardized artificial laboratory assays, such as toxicological experiments. These assays use standard incubation water or commercially available resources and taxa and, thus, they are highly replicable and very useable for explanatory studies of the mode of action of different stressors. However, opposed to field experiments, standardized artificial assays lack realism as the experimental conditions largely differ from those in the ecosystems of interest and, therefore, results may not be so easily extrapolated directly to nature. Thus, researchers need to choose between realism and high reproducibility of experiments (Fig. 1).

The optimal experiment should offer highly extrapollable responses while being highly replicable (i.e., top-right area of Fig. 1). Researchers have been using an intermediate experiment type between field experiments and standardized artificial laboratory assays: the *model ecosystem approach* as described by Landner *et al.*, (1989) (represented as the dashed box in Fig. 1). The aim of the model ecosystem approach is to mimic the target ecosystem in order to observe the response to a potential stress in the model system and to extrapolate to nature (Landner *et al.*, 1989). Model system can never be identical to the real world and, therefore, they will always be approximations of the target system (Landner *et al.*, 1989). The model ecosystem approach is common in many experiments that are performed in microcosms and mesocosms. The scientific literature shows a diverse set of experiments that following this approach and focusing on aquatic ecosystems have proven the effect of several variables of interest, such as food quality (LeRoy *et al.*, 2007; Lecerf & Chauvet, 2008; Casas *et al.*, 2013), shredder taxa (Taylor & Chauvet, 2014) or mesocosm size (Uiterwaal & Delong, 2018). However, many of those studies try to mimic in such an extent the target system (e.g., the use of water, food and taxa collected in the field), that the very desirable comparability among experiments is challenged.

Thus, when deciding the experimental conditions, researchers need to tradeoff between transferability to specific ecosystems and comparability with experiments previously published. One of the variables that researchers need to make a decision upon is incubation duration. It is a variable that does not play in the tradeoff between the transferability and the comparability, and yet, it plays an important role explaining the response of the variables in many studies (e.g., Cruz-Rivera and Hay,

2000; Lieske and Zwick, 2007). The relevance of exposure time was long ago described by Haber (1924), who pointed out that concentration and exposure time to toxicants affect the response to toxicity, what led to the so-called Haber´s rule, which states that exposure time amplifies the responses and less severe impacts take longer to be able to be detected. Additionally, measured response variables differ in the required response time; usually sub-individual physiological changes show fast responses to environmental changes (i.e. stress signs). Among the variety of experimental conditions, incubation duration can be easily standardized to increase comparability among assays without losing realism in the response (Time standardization, Fig. 1), but no effort on finding a standardized and optimum incubation time when working with the model ecosystem approach (i.e. microcosms / mesocosoms) seems to arise from the available literature.

Together with the experiment type and incubation conditions, researchers need to decide which response variables to measure. Here again there is a tradeoff between early stress signals and effects measured at

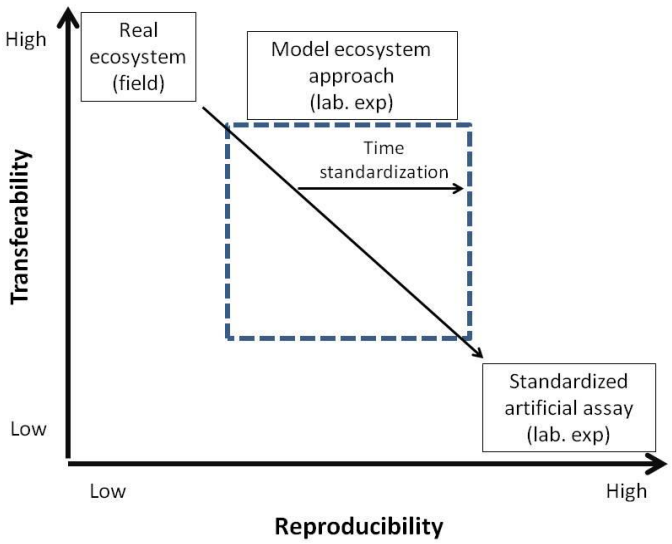

Fig. 1. Schematic representation of the characteristics of different experiment types.

ecosystem level. Biomarkers can be used as early warning signals and are expected to precede those changes occurring at higher levels of biological organization (Depledge & Fossi, 1994). Therefore, they can be used in a predictive way allowing to detect changes before irreversible environmental damage of ecological consequences occur at ecosystem level. However, few studies have tried to find parallelism between biomarkers and ecosystem level responses.

…to assess the effect of food quality…

The proposed microcosm experiments are useful to assess several impacts affecting river ecosystems. In low-order headwater streams, where light is limited by natural riparian forest, ecosystems rely on allochthonous leaf litter as primary source of energy (Webster & Benfield, 1986; Cummins *et al.*, 1989). In freshwater ecosystems huge quantities of organic carbon are processed (~ 2.7 Pg C

yr^{-1}, Tranvik et al., (2009)), which contribute substantially to the global carbon fluxes (Battin *et al.*, 2009). Thus, leaf litter decomposition is a key ecosystem process that consists on nutrient mineralization, incorporation of organic carbon and nutrients into decomposer biomass, and transfer of nutrients and energy to higher trophic levels (Gessner, Chauvet & Dobson, 1999). Nevertheless, plant species from the riparian forest produce litter that can greatly differ in their chemical composition, such as in the carbon to nutrient ratios (C:nutrient) or concentrations of secondary compounds (e.g., phenols, tannins; Berg and Meentemeyer, (2002)). One species that can be considered key for the functioning of temperate freshwater systems is alder (*Alnus* sp.). Species of this genus present leaf litter of elevated nitrogen concentration (2.16 % of dry mass Boyero et al., (2017) and a C:N ratio of 25.4, Kominoski, Marczak & Richardson (2011)) and low concentration of secondary metabolites (tannin concentration 1.51% of dry mass, Boyero et al., (2017)). Those traits make *Alnus* highly palatable to stream detritivores (Graça *et al.*, 2001). Other tree species growing in the surroundings of water courses such as *Fagus* or *Quercus*, present two and three times lower nitrogen concentrations and 8 and 10 times higher tannin concentrations, respectively (Boyero *et al.*, 2017). Those leaf litter quality traits show tight relationship with litter breakdown rates in stream (Lecerf & Chauvet, 2008; Hladyz *et al.*, 2009; Schindler & Gessner, 2009).

Globally, the area occupied by plantations has significantly increased during the last decades (Payn *et al.*, 2015). The replacement of native riparian forest by monoculture plantations, native or introduced species, have directly affected freshwater ecosystems due to: a) their elevate dependence on riparian leaf litter as a source of energy (Minshall, 1967), b) the preference of invertebrate consumers towards some litter types (Graça, 2001) and c) the effects of the quality and quantity of ingested material on the body condition and fitness of the consumer (Gee, 1988; Kendrick & Benstead, 2013; Larrañaga, Basaguren & Pozo, 2014). In headwater streams with dense and highly diverse riparian forests, leaf litter appears in batches of mixed leaves along the streambed. Decomposition rates of those complex packs are not always predictable from single species dynamics (Kominoski *et al.*, 2007; Schindler & Gessner, 2009; Ferreira, Encalada & Graça, 2012). High quality deciduous leaf have promoted litter decomposition in several experiments (e.g., Kominoski et al., 2011; Lecerf et al., 2005; McKie and Malmqvist, 2009). The observed stimulation could be explained by the fact that herbivores maintain mixed diets in order to acquire the required nutrients (complementarity) or to dilute toxicity of some compounds (Freeland & Janzen, 1974; Pennings, Nadeau & Paul, 1993; Bernays *et al.*, 1994). Even if processing rates of litter mixtures have been widely studied (e.g., Ferreira et al., 2012; Schindler and Gessner, 2009; Taylor et al., 1989), the performance of consumers in the context of litter mixtures has been less studied (but see Flores et al., 2014). Nonetheless, it is well known that the quality and quantity of the ingested leaf litter have direct consequences on consumer´s performance; growth rates (Mulder & Elser, 2009; Kendrick & Benstead, 2013) and the capacity to accumulate biomolecules (e.g., lipids and protein) depends on the consumed material quality (Gee, 1988). Huge amount of energy is required both for growth and reproduction (Beer-Stiller & Zwick, 1995) and the energy invested on one of them is not available for the other with direct consequences for the fitness. When high quantity and quality resources are available, as *Alnus* leaf litter, individuals are able to

store fats and therefore more energy can be derived to reproduction, affecting the development of the entire population (Kley & Maier, 2003). In this sense, Larrañaga et al., (2009) observed that *Echinogammarus berilloni* individuals showed significantly higher densities and body condition in streams surrounded by native forests with riparian forests rich on *Alnus* (with only 20.5 % of the total litter inputs being *Alnus*; Pozo et al., 1997) than in streams surrounded by eucalypt plantations (where 0.3 % of the total litter imput was *Alnus*). Although differences among consumers feeding on monospecific leaf litter diets have been studied extensively, it is not clear, nevertheless, the degree of improvement that a relatively small contribution of *Alnus* can create on them.

…and chemistry of the water…

During the last decades, the concentrations of nutrients, organic pollutants and toxic chemicals has increased in water bodies as a consequence intensive agricultural and industrial activities (Lacorte *et al.*, 2006). The complexity of those chemical cocktails released by the society is increasing and the biological effects are unpredictable (Sabater, Elosegi & Ludwig, 2018). In order to reduce loads of nutrients, organic matter and other pollutants, many wastewater treatment plants (WWTP) have been built. However, they still consist a major point source pollution to many freshwater ecosystems (Carey & Migliaccio, 2009; Munz *et al.*, 2017). The effluents released from WWTPs consist of complex mixtures of nutrients, organic matter, metals, and other pollutants, including some that have specific regulation in many parts of the world, but many others that are not regulated (so called emerging pollutants) (Petrovic *et al.*, 2002; Gros, Petrović & Barceló, 2007). Some of these compounds are toxic (e.g. pesticides) and decrease biological activity, while others (e.g. nutrients), can subsidize the biological activity when they appear at low concentrations (Martí *et al.*, 2001; Ribot *et al.*, 2012) or stress it if they are present at high concentrations. This response pattern has been coined 'subsidy-stress response' (Odum et al. 1979). Works that have focused on the microbial community clearly explain how the 'subsidy-stress' pattern can appear when dealing with complex mixtures of pollutants: a moderate increase in nutrient concentrations stimulates microbial activity, biomass and even fungal diversity (Pascoal & Cássio, 2004; Ferreira, Gulis & Graça, 2006; Duarte *et al.*, 2009; Fernandes *et al.*, 2014), but the effect of pesticides dominate at higher concentrations of mixed pollutants and negative effects have been described on fungal leaf decomposers (Gardeström *et al.*, 2016). Although the contribution of the microbial community to the decomposition process is lower than that of detritivore invertebrates (Hieber & Gessner, 2002b), they play an important role on the transformation of the quality of the leaf litter. Particularly fungi increase the nutrient content of leaves and degrade leaf recalcitrant structural components making leaf litter more palatable and nutritious for shredders (Bärlocher, 1985). Apart from changes at the microbial community level, contaminants derived from the effluent can also accumulate in leaf materials (Dimitrov *et al.*, 2014) and therefore cause toxic effects when ingested together with leaf litter (Bundschuh *et al.*, 2013), and thus, affect macroconsumers. In streams receiving low quantities of WWTP effluents, shredders might benefit from the higher quality of the available benthic organic matter due to the higher activity of the microbial community. However, at higher effluent concentrations invertebrates should suffer both, direct toxic effects from the water column and indirect toxic effect derived from the ingested toxic compounds

in the resource. This way, the effects of the effluent on the macroconsumers should, as for the microbial community, be dependent on the chemical characteristics of the effluent and the degree of dilution of the effluent in the stream.

…and sediments.

We have observed that two compartments of the stream ecosystem (food resource and water), water and resource, can be altered as a consequence of anthropogenic impacts having direct effects on the performance of detritivores and thus, the entire food web. We should not forget, nevertheless, that the substrate or the sediment the organisms leave within or on top can also interact with water and resources. This sediment can act as a sink as well as a potential long-term source of different contaminants in aquatic ecosystems. For instance, many persistent organic pollutants (POPs), such as PCBs, are adsorbed to organic matter and particles in the sediments increasing their persistence in natural systems, which is most of the time assisted by the hydrophobic nature and low solubility in water that these compounds show (Nizzetto *et al.*, 2010). As a consequence, biota is often exposed to complex mixtures of pollutants that have not entered recently the ecosystem and are legacies of the past (Sprovieri *et al.*, 2007; Barakat *et al.*, 2013). For this reason, even if all compounds we can analyse from the water or sediment may appear below their toxicity levels, toxic responses may manifest from chemical mixtures and relatively high baseline toxicity levels (Hermens *et al.*, 1985; Escher *et al.*, 2002). Within pollutants mixtures, each compound might have a specific effect on the biota but the understanding of the net effect of mixtures is still low and therefore, we will try to improve the knowledge by assessing the overall effect of mixtures at different biomarkers on the amphipod *Monoporeia affinis*.

The present PhD focuses on model ecosystem approach using microcosm laboratory experiments. The effects of the incubation time, resource quality, water quality and sediment contamination on freshwater detrital systems are described.

Structure of the PhD

Four groups of questions articulate the chapters of the PhD:

1) How does the incubation duration affect the responses of microcosm experiments? Do different variables require a different response time? Can we use some variables as early warning signals because they precede responses occurring later in time?
2) Do changes in resource quality affect processing rates and consumer performance? Does the addition of a high quality resource have stimulating effect on processing rates and consumer performance?
3) How does the WWTP effluent affect the stream ecosystem via changes in food and water quality? Is the response related to the concentration of the effluent?
4) How does the biota respond to the pollution of the sediment?

This PhD has been structured in 4 chapters. The first chapter focuses on the importance of incubation duration on the response to differences in resource quality. Five leave species of contrasting quality were used for the experiment in the laboratory: *Corylus avellana* (L.), *Alnus glutinosa* ((L.) Gaertn), *Fraxinus excelsior* (L.), *Quercus robur* (L.), and *Fagus sylvatica* (L.). Samples were taken over time (sampling days: 2, 4, 8, 16 and 32) in which *Echinogammarus berilloni´*s (Catta) consumption rates and performance

(RNA:BM, growth rate, mass body condition and lipid body condition) were measured. The second chapter studied the effect of the availability of a high-quality resource (alder) following the same experimental design as in chapter one. In each microcosm an alder disk together with four disks of one of the other species were offered. The stimulating effect of alder disk addition was assessed comparing responses with and without alder. The third chapter included both, field manipulative experiment (following a BACI design) and laboratory experiment. For the field experiment *E. berilloni* individuals were incubated upstream and downstream of a WWTP effluent discharge point before and after the beginning of the effluent discharge. Samples were taken after 8 and 15 days of incubation. At the laboratory microcosm experiment, the effect of the concentration of the WWTP effluent was assessed with a microcosm experiment in which we compared the microbial and consumer performance and organic matter processing rates in a range of effluent dilutions (0, 20, 40, 60, 80 and 100 %). Samples were taken after 8, 15 and 30 days of incubation in order to assess the effect of incubation duration. Both in the field and the laboratory experiment, microbial breakdown and microbial AP and BG exo-enzymatic activity and respiration rate and amphipod consumption rates and performance (RNA:BM and growth) were measured. The forth, and the last chapter, was carried out during a stay at Stockholm University and assessed the effect of a mixture of chemicals on *Monoporeia affinis* (Lindström, 1855) individuals. In this case, exposure to the mixture of chemicals (hexachlorobenzene, biphenyl, PCB52, PCB101, PCB153, pyrene and phenanthrene) was via both food and sediment contamination. Two exposure scenarios were tested; "High-Peak exposure" which consisted on a high chemical concentration exposure during 4 days and "Chronic exposure" where individuals were exposed to lower chemical concentrations for 22 days. The response of several biomarkers (ORAC, TBARS, AChE, RNA:DNA, RNA:P and DNA:P) and the correlations between them were assessed.

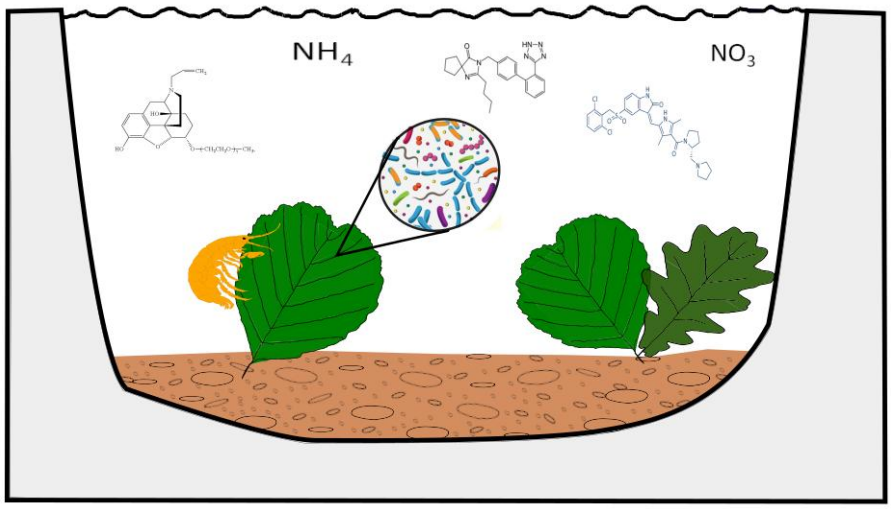

Fig 2. Simplified respresentation of the studied microcosm compartements at different chapters.

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Chapter 1

Laboratory incubation time creates inconsistent consumption and performance responses in the amphipod *Echinogammarus berilloni*

Libe Solagaistua, Arturo Elosegi and Aitor Larrañaga

Abstract

The model ecosystem approach offers realistic responses by mimicking the target ecosystem on microcosm experiments, however, the comparability among studies is not straightforward as they may differ in many experimental conditions. In order to increase comparability between microcosm experiments, incubation duration could be standardized without loosing realism in the response. On the other hand, some response variables being tested in microcosm studies can respond fast (early warning signals) and are believed to precede changes occurring later in time at higher levels of biological organization. In this experiment we test the effects of resource quality and incubation duration on the performance of a freshwater macroinvertebrate. The amphipod *Echinogammarus berilloni* (Catta.) was fed with five leaf species of contrasting quality and we analysed the responses (consumption rate, death rate, RNA:BM, growth rate mass body condition and lipid body condition) in various moments over time (sampling days: 2, 4, 8, 16 and 32). We predicted that i) resource quality would affect all the response variables being tested, ii) consumption rate and RNA:BM ratio would act as early warning signals and iii) the inter-individual variation would gradually reduce with time. In our study, resource quality affected consumption rates, although the correlation was not straightforward; *Fraxinus* was the most consumed leaf despite not being identified as the highest quality resource. Amphipod body mass condition was significantly affected by the resource quality but the rest of the measured variables in the consumer were not. We observed incubation time to be an important factor affecting the response; three variables included time as significant explanatory factor in the model and we observed significant interactions between resource quality and incubation duration, which shows the difficulty when comparing studies performed at different incubation times and highlight the need of time standardized experiments. Consumption rate and RNA:BM ratio did not act as early warning tools, as they did not precede changes on amphipod performance or did not significantly respond to resource quality. The predicted reduction in variability with time was observed only for consumption and growth rate. Our results pointed that the responses in the first 8 days of incubation were more erratic (higher internal variability) and, thus, a minimum incubation duration of 16 days can be recommended.

Keywords: Microcosm experiment, incubation time, resource quality, consumption, amphipod performance

1. Introduction

Freshwater ecosystems are affected by multiple stressors derived from global environmental change, including nutrients and pollutants, land use changes and geomorphological disturbances (Sabater *et al.*, 2018). Defining the effects of those stressors is a major scientific challenge that has been approached from diverse perspectives. On one hand, observational field studies (e.g., Ponsatí et al. 2016) can offer information on the effects of these stressors on real conditions, but are strongly limited in the attribution of causality. Manipulative field experiments, especially those following a BACI design (e.g., Arroita et al. 2016), overcome some of the problems of observational studies, but can face the idiosyncratic nature of the study sites making difficult generalisations. On the other extreme, standardized artificial assays, such as ecotoxicological assays, use standard incubation water or commercially available resource and taxa. Those assays are highly replicable an allow attributing causality as well as an understanding of the mechanisms of stress and resistance. In these assays, procedural details can strongly affect the outcome of toxicological assays, and thus, has led authors to strive for a standardization of their procedures. For instance, the "OECD Guidelines for the testing of chemicals" is a collection of internationally agreed testing methods for the selection and ranking of candidate chemicals in terms of toxicity and effects on natural systems (https://doi.org/10.1787/20745761). Following these guidelines, a variety of studies focus on understanding how each impact affects specific taxa and on how different stressors are ranked (e.g., Dahl et al. 2006; Lebrun et al. 2017). However, incubation conditions are usually far from the target ecosystem in the standard artificial assays and thus, responses are hardly extrapolated to the ecosystem scale. This fact has led to devise the model ecosystem approach (Landner *et al.*, 1989), which is located between standardized artificial assays and field experiments and can be referred to as microcosm or mesocosm studies. The aim of the model ecosystem approach is to mimic the target ecosystem (e.g., the use of water, food and taxa collected in the target system) in order to observe the response to a potential stress in the model system. Nevertheless, even if the number of published microcosm assays based on model ecosystem approach is growing fast, the comparability among them is very small. For example, Rasmussen et al. (2012) assayed the effect of a triazole fungicide and a pyrethroid insecticide, and the combination of both, on microbial decomposition, microbial biomass and shredding activity. Zubrod et al. (2015) tested the effect of a mixture of fungicides (one of them also a triazole) via waterborne exposure and resource, due to co-ingestion of the fungicide and shift in leaf-associates fungal community and the consequent changes in resource quality. Both assays followed a model ecosystem approach and aimed at assessing the effect of fungicides on invertebrate shredding activity but they differed in experimental conditions such as water temperature, food offered and taxa studied. In addition to these experimental conditions, the incubation unit, incubation duration or the photoperiod can also differ among studies. It has already been shown that temperature (Webster & Benfield, 1986; Menéndez, Hernández & Comín, 2003), food quality (LeRoy *et al.*, 2007; Lecerf & Chauvet, 2008; Casas *et al.*, 2013), shredder taxa (Taylor & Chauvet, 2014) and mesocosm size (Uiterwaal & Delong, 2018) affect the responses and therefore, the comparability between those studies is not straightforward.

In microcosm experiments based on model ecosystem approach, the use of taxa, food or stream water from selected local ecosystems increases the capacity to extrapolate results obtained in the assay to those local systems but reduces comparability among studies. Thus, a trade-off between realism and comparability with previous assays has to be considered whenever a microcosm experiment is designed. Specimens coming from natural populations or from standard commercial cultures can contrast in their response in laboratory experiments. Individuals grown up at laboratory cultures usually are from the same life stage and present similar body condition and energy requirements, therefore, their response to experimental conditions tends to be very homogeneous. However, conspecific individuals collected in the field may differ in many traits including gender, age, size, behaviour or physiology (Bolnick *et al.*, 2011), even in stoichiometry (Cai et al. 2016), which can strongly affect their performance under experimental conditions, making the responses less homogeneous albeit more realistic. On the other hand, incubation duration is one of the factors that can be standardized in microcosm assays. Given that incubation time is a variable every researcher needs to decide upon, it is surprising that few studies address its effect on the consistency of the responses.

Besides the impact severity, the selection of response variables is critical, as each one of them require a different time span to manifest a response. Certain variables respond fast to changes in their environment and thus, they will provide early warning signals of the biological effects. Those early warning responses can precede the responses occurring later in time at higher levels of biological organizations. In stream ecosystems, leaf litter quality traits determine the breakdown rates (Lecerf & Chauvet, 2008; Hladyz *et al.*, 2009; Schindler & Gessner, 2009) and ingested resource quality and quantity will have direct consequences on the consumers performance. In the recent years, it is becoming increasingly common to assess individual metabolic status by using RNA-based measurements. The rationale of using RNA-based ratios consists on the fact that the RNA content of whole organisms is primarily ribosomal RNA. Consequently, concentration of RNA is directly related to the protein synthesis (Elser *et al.*, 2000b). DNA, on the other hand, is accepted as an surrogate of number of cells (Buckley, Caldarone & Ong, 1999). Consequently, RNA:DNA, or RNA:BM, ratio is a proxy of growth and metabolic status and has been shown to be a good and very responsive indicator of nutritional condition (Stuck, Watts & Wang, 1996; Wagner, Durbin & Buckley, 1998). Some works report that changes in food quality correlate with growth and RNA (Acharya *et al.*, 2006); RNA:DNA ratios of starved-fed postlarvae rapidly decline between the first and second day of starvation and continued to gradually decrease through Day 12. Once feeding is resumed, RNA:DNA ratios immediately begin to increase (Stuck *et al.*, 1996). Similarly, a clear decline of total RNA has been also observed after 4 days of fasting at 8 ºC on the shrimp *Mysis relicta* (Schlechtriem *et al.*, 2008). Individual growth and body condition also respond to resource quality (Larrañaga *et al.*, 2014) but time required to observe a response is supposed to be longer than for RNA-based ratios. For instance, Delong et al., (1993) offered different food resource with varying nutritional values to *Gammarus fasciatus* in a laboratory experiment and no difference in growth rates were distinguished among food types until the third incubation week. From week 4 to 6 differences were significant. Hervant et al. (1999) pointed out that during starvation of two aquatic crustaceans, total lipid and triglyceride content were reduced only after 150 days.

However, during re-feeding, lipid stores returned to control levels within 15 days. It is then expected that each variable will need more or less time to respond to an environmental condition, and thus, will give us meaningful information after different microcosm incubation times.

Our objective here was to test the effects of stoichiometric imbalance and incubation duration on the response of freshwater invertebrates. We fed the amphipod *Echinogammarus berilloni* (Catta.) with five leaf species of contrasting quality and analysed the responses (consumption rate, death rate, RNA:BM, growth rate mass body condition and lipid body condition) over time. We predicted that, i) resource quality would affect the response, ii) consumption rates and RNA:BM ratios would act as early warning signals and will precede changes of amphipod growth rate and body condition, which will respond later in time, and iii) the inter-individual variation would gradually reduce with time due to constant microcosm conditions and, as a consequence, the power to find differences among treatments would increase with time.

2. Materials and methods

2.1. Organic matter and experimental taxa

We used leaf litter of five common riparian tree species in European streams, chosen because they differ on traits such as nutrient content or toughness (Boyero *et al.*, 2017). *Alnus glutinosa* ((L.) Gaertn), *Corylus avellana* (L.), *Fraxinus excelsior* (L.), *Fagus sylvatica* (L.) and *Quercus robur* (L.) were collected near Bilbao (North Iberian Peninsula 43º15´47´´ N 2º56´06´´ W). Leaves were collected after abscission in October 2015 and disks (12 mm diameter) were punched using a cork borer, air dried and stored in 24-well culture plates in a dark dry place until further use. Leaf litter elemental analyses were performed in 5 replicates per species after grinding. Nitrogen and carbon content were analyzed with Eurovector EA 3000 CNH analyzer (Eurovector, Milan Italy). Phosphorus and potassium were analyzed after acid digestion (0.5 g dry mass in 15 mL of HNO_3 69%) in an optical emission spectrophotometer with inductively coupled plasma (ICP-OES, Horiba Jobin Yvon, Activa). Leaf toughness was measured using a penetrometer with a 0.49 mm^2 area steel rod (Boyero *et al.*, 2011a) (15 replicates per species).

As consumer macroinvertebrate we chose the amphipod *Echinogammarus berilloni* (Catta.) as it is one of the most abundant species in the streams of the region (Larrañaga *et al.*, 2009a). Additionally, it is extensively used in laboratory experiments due to its wide distribution and the great contribution to organic matter processing (Larrañaga *et al.*, 2009a). Specimens were kick-sampled (500 µm mesh size) in Guriezo (Adino Stream, 43º20´27´´ N 3º20´20´´ W), individually enclosed in 30 mL containers with pores for water interchange and carried to the laboratory immersed in transport boxes filled with stream water. Animals with a first thoracic segment length between 0.24 and 1.26 mm were selected (4.59 and 17.08 mm in total body length), but breeding females were discarded as they show a clearly differentiated biochemical composition (Larrañaga *et al.*, 2009b). The animals were acclimatized in a controlled temperature room (15 °C) without food for 3 d prior to the experiment.

2.2. Experimental setup

We prepared a set of microcosm, consisting of test tubes (2 cm diameter and 20 cm long, enough to allow free movement of the animals) with 40 mL of filtered (100 µm nylon mesh) stream water (from the same place amphipods were collected) and 5 leaf disks of one species. Hereafter, the

name of the incubated leaf disks in the microcosm will correspond to the treatment name (i.e., *Corylus*, *Alnus*, *Fraxinus*, *Quercus* and *Fagus*). Prior to the experiment, the groups of 5 leaf disks were conditioned in stream water at the laboratory microcosms for 15 days at 15 ºC, 12:12 light photoperiod and under constant aeration (water renewed at day 7). The conditioning was aimed at reducing the leachates and promoting microbial colonization. After those 15 days, water was renewed again, an individual of *E. berilloni* was added per microcosm and the rest of experimental conditions were kept as during conditioning. The experiment lasted for 32 days and water was renewed at days 8, 16 and 24 to avoid accumulation of deleterious substances and shortage of nutrients for microbes. Water samples were collected at each renewal and filtered through pre-combusted glass-fiber filters (Whatman GF/F, 0.7 µm pore size) and stored at - 20 ºC until analysis. The concentration of soluble reactive phosphorus (SRP) (molybdate method (Murphy & Riley, 1962)) and ammonium (salicylate method (Reardon, Foreman & Searcy, 1966)) in water was determined colorimetrically on a UV-1800 UV-Vis Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The concentrations of chloride (Cl), sulfate (SO_4) and nitrate (NO_3) anions were determined with capillary ion electrophoresis (Agilent CE) (Environmental Protection Agency, 2007). Microcosms were destructively sampled at days 2, 4, 8, 16 and 32, with 20 replicates per treatment (20 replicates x 5 leaf species x 5 sampling times, total 500 individuals).

2.3. Response variables

We measured six response variables, one related to ecosystem functioning (organic matter consumption) and four related to the performance of the amphipod (death rate, growth rate, mass body condition, lipid body condition and RNA:Body mass ratio). Death rate was expressed as the natural logarithm (ln) of the difference between initial and final number of individuals alive per treatment and sampling day, standardized with incubation duration.

2.3.1. Consumption rates

The five air-dried disks of each microcosm were weighed before conditioning (air dry mass). At each sampling day, disks were retrieved from the microcosms, oven dried (70 ºC, 72 h) and ashed (500 ºC, 4 h) to calculate the ash free dry mass (AFDM). Mass loss during conditioning was measured and used to estimate the initial AFDM. Consumption rates were calculated from the difference between estimated initial dry mass and final weighed dry mass of the 5 disks in each microcosm, and standardized by the geometric average body mass of the individual and total incubation time (mg AFDM · mg BM^{-1} · d^{-1}). An additional set of 9 microcosms per leaf species were incubated without amphipods to estimate microbial decomposition rates and therefore correct consumption rates.

2.3.2. Growth rate

Amphipods were photographed at the beginning and end of the experiment with a binocular microscope (Leica M165FC, Wetzlar, Germany). The dorsal length of the first thoracic segment (*DL*) was measured from photographs using the "Leica Application suite V4" program (LAS V4.1). Initial and final total body lengths (*BL*) were calculated using Eq. (1) (Flores *et al.*, 2014a). Instantaneous growth rate (*IGR*) for each individual was calculated using Eq. (2) where t is the incubation duration, BL_t is the body length at time t and BL_0 is the initial body length (Flores *et al.*, 2014a).

Eq. (1) $BL = 14.458 \cdot DL - 0.110$; (mm)

Eq. (2) $IGR = (\ln(BLt) - \ln(BL_0)) / t$; (mm · d^{-1})

2.3.3. Body condition: mass and lipids

By means of the Eq. (3) (Flores *et al.*, 2014a) the body mass (*BM*) was estimated at the end of the experiment, and this value was used as the descriptor for each invertebrate dry mass in the experiment.

Eq. (3) $BM = 0.8213 \cdot BL - 4.3025$; (mg)

Half of the sampled amphipods (10 replicates per leaf species and sampling time) were freeze dried and weighed. We compared the dry mass obtained this way with the expected body mass calculated from Eq. (3) to be used as a proxy of mass body condition. The mass body condition was logaritmized (log10) before statistical analyses; a value of 0 indicates that the weighed mass exactly matches what is expected from the body length, while positive and negative values show heavier or lighter than expected individuals, respectively. Total individual lipid content was measured as a proxy of energy reserves stored. Lipid amount was quantified in freeze-dried entire individuals. Extraction was performed incubating individuals in diethyl ether at 4 ºC for 2 d and sonicated twice for 15 min (Bandelin Sonorex; Bandelin Electronic GmbH and Co., Berlin, Germany). Extraction was followed by digestion with H_2SO_4 (200 ºC) and quantification by spectrophotometer. Cholesterol was used as standard. Lipid body condition was expressed as Lipid · mg BM^{-1}.

2.3.4. Nucleic acid content

The rest of the individuals (10 replicates per leaf species and sampling time) were stored in RNAlater solution (Ambion) until further nucleic acid analysis. Microplate fluorometric high-range assay with Ribo-Green was performed to quantify RNA after extraction with *N*-laurylsarcosine followed by RNase digestion as described in detail by Gorokhova and Kyle (2002). Fluorescence measurements were performed using a Tecan GENios microplate reader (Cavro Scientific Instruments, Sunnyvale, filters: 485 nm excitation and 520 nm emission). Measurements were performed in black solid flat-bottom microplates, scanned during 0.2 s · $well^{-1}$, with 10 measurements per well at constant temperature (37 ºC). Measured RNA concentrations were expressed as µg RNA · mg BM^{-1}.

2.4. Data analysis

A high degree of correlation was observed among leaf traits (minimum R^2 in pairwise Pearson correlation: 0.67; supplementary material A). Thus, we integrated all variables in an index of leaf litter quality, calculated by means of Eq. (4), where *T* is the measured value for the trait and *n* the number of measured traits.

Eq. (4) $Quality\ index = 1 - ((T_1 / (n \cdot T_{1(max)})) + \ldots + (T_n / (n \cdot T_{n(max)})))$

We fitted Gaussian models (Madsen & Thyregod, 2010a; Zuur & Ieno, 2010b) to test for the effect of leaf species (1st prediction) and incubation duration on the response variables (consumption, death rate, RNA:BM, growth rate, mass body condition and lipid body condition). For parameter estimation, generalized least squares (Pinheiro & Bates, 2000) were used, via the gls() function of the package nlme (Pinheiro *et al.*, 2018). The fixed structure of the model included leaf species (fitted as a discrete explanatory variable, with five levels, *Corylus*, *Alnus*, *Fraxinus*, *Quercus*, *Fagus*), incubation duration (fitted also as a discrete explanatory variable, with five levels, Day 2, Day 4, Day 8, Day 16 and Day 32) and the interaction between both sources of variation. AIC comparisons revealed the

need to add a variance structure to the models to deal with heteroscedascity, which allowed different variances per day (varIdent(form= ~1|Day)). No heteroscedascity was observed among leaf species. As both consumption and growth rates presented leptokurtic distributions, data were normalized via the gaussianize() function of the package LambertW (Goerg, 2011). To check how each response variable responded over incubation duration (2nd prediction) analyses of amphipod consumption and performance were carried out first using data from all sampling days and later with data from each sampling day separately. When using data from all sampling days, generalized least squares gls() were used where the fixed structure of the model consisted on leaf species (fitted as explanatory variables) and incubation duration included as a block factor. We added a variance structure to the models to deal with heteroscedascity, which allowed different variances per day (varIdent(form= ~1|Day)). No heteroscedascity was observed among the different leaf species. When using separate data from each sampling day, linear models lm() were used where the fixed structure of the model consisted on leaf species (fixed as explanatory variable). As both consumption and growth rates presented leptokurtic distributions, data were normalized as explained above.

Variance reduction over time was tested with linear models lm() from package nlme (Pinheiro *et al.*, 2018) for each response variable (3rd prediction). For the different response variables, we computed first the standard error for the variables of interest for each leaf species and day, and then we created models with the standard error as response variable, incubation duration fitted as a discrete explanatory variable and species as random factor. The critical alpha value for all analyses was 0.05 and all statistical analyses were conducted using R statistical software (version 3.1.2, R Core Team 2018).

3. Results

Stream water used over the experiment ranged in phosphorus concentration from 4.7 $\mu g \cdot L^{-1}$ to 28.2 $\mu g \cdot L^{-1}$ and in ammonia concentration from 35.6 $\mu g \cdot L^{-1}$ and 106.7 $\mu g \cdot L^{-1}$. The mean values of Cl, SO_4 and NO_3 were 11.4 $mg \cdot L^{-1}$, 7.9 $mg \cdot L^{-1}$ and 2.4 $mg \cdot L^{-1}$ respectively.

The five leaf litter species significantly differed in stoichiometry (C:N, C:P, C:K ratios), in toughness and, consequently, in their overall leaf quality index (Table 1). *Corylus* showed the highest leaf quality index, with the highest concentration of phosphorus and potassium per carbon and lowest toughness. *Alnus* and *Fraxinus* only differed in nitrogen content as the former presents the highest concentration. The leaf species with the worst quality was *Fagus* with the lowest nitrogen proportion per carbon. Even if the overall quality was better for *Quercus* than for *Fagus*, the former had lower concentrations of phosphorus and potassium per carbon and higher toughness (Table 1).

Consumption rate was affected by both leaf species and incubation duration (Species and Day, $p < 0.001$, Table 2, Fig. 1). Consumption rates were significantly affected by leaf quality (supplementary material B; Quality, $p < 0.001$). However, even if it was not the highest quality leaf, *Fraxinus* was the most consumed leaf species, with 0.594 mg AFDM · mg BM^{-1} · d^{-1} consumption at day 2 and 0.235 mg AFDM · mg BM^{-1} · d^{-1} at day 32. The less consumed leaf species were *Quercus* at day 2 and *Fagus* at day 32, with mean consumption rates of 0.084 and 0.079 mg AFDM · mg BM^{-1} · d^{-1} respectively. Overall, the consumption rate decreased over incubation duration for all leaf species

Table 1. Sumary of the characteristics of the materials used in the experiment. Nitrogen, phosphorus and potassium to carbon ratios (n = 5) and leaf toughness (kPa) (n = 15) values are shown. Mean values and standard error are presented. Non-matching letters indicate statistically different groups (Tukey´s test, $p < 0.05$).

Leaf litter sp.		
C:N		
	Corylus	19.92 ± 0.38^{a}
	Alnus	$14.05 \pm 0,07^{b}$
	Fraxinus	22.76 ± 0.66^{c}
	Quercus	37.39 ± 0.40^{d}
	Fagus	52.70 ± 0.11^{e}
C:P		
	Corylus	838.69 ± 11.20^{a}
	Alnus	1141.87 ± 20.65^{b}
	Fraxinus	1128.87 ± 31.03^{b}
	Quercus	3259.20 ± 84.56^{c}
	Fagus	2073.13 ± 69.22^{d}
C:K		
	Corylus	1157.70 ± 75.88^{a}
	Alnus	1920.94 ± 97.92^{b}
	Fraxinus	1719.30 ± 143.15^{b}
	Quercus	2804.83 ± 75.40^{c}
	Fagus	2729.15 ± 66.74^{c}
Toughness (kPa)		
	Corylus	57.81 ± 3.09^{a}
	Alnus	70.47 ± 4.96^{ab}
	Fraxinus	81.52 ± 6.51^{b}
	Quercus	123.48 ± 8.53^{c}
	Fagus	$107.4\ 3\pm 5.04^{c}$
Quality index		
	Corylus	0.621
	Alnus	0.532
	Fraxinus	0.487
	Quercus	0.130
	Fagus	0.073

Most importantly, the interaction between leaf species and incubation duration was significant (Species:Day, $p = 0.001$, Table 2, Fig. 1), showing that the consumption of different leaf species did not show the same pattern over the experiment. For instance, at sampling day 8 consumption rates of *Corylus*, *Alnus* and *Fraxinus* did not differ whereas at sampling day 32, the differences between treatments become clearer.

In total, 83 out of 500 individuals died during the experiment, but neither the leaf species nor the incubation duration affected death rate (Species, $p = 0.242$; Day, $p = 0.491$, Table 2). The RNA:BM ratio was not affected by leaf species nor incubation duration, although the interaction between leaf species and incubation duration was significant (Species:Day, $p = 0.044$, Table 2, Fig. 2). For instance, at day 2, the RNA:BM ratio followed a hump-shape response along the leaf quality gradient, whereas at day 8 the response was inversed (Fig. 2). Leaf species did not significantly affect growth but incubation duration did (Day, $p = 0.009$, Table 2, Fig. 2). The overall amphipod mass body condition was affected by leaf species and incubation duration (Table 2, Fig. 2). The interaction between both sources of variation was significant, which

means that mass body condition of individuals fed with different leaf species ranked differently over the duration of the incubation (Table 2, Fig. 2). Individuals fed with low quality resources (in this case *Fagus*) presented the best mass body condition at the second sampling day and the worst at day 16. However, amphipod lipid body condition was neither affected by leaf species nor incubation duration, and the interaction was also non-significant (Table 2, Fig. 2).

As predicted (2nd prediction) consumption rate was significantly affected by leaf species from the second day on (Table 3). Consumption rate showed a very consistent pattern, as it was significantly affected by leaf species when considering all the incubation times and also when analysing sampling days separately (Table 3). However, contrary to expected, RNA:BM did not respond to the stoichiometric imbalance at any of the sampling days. Regarding amphipod body condition, the mass body condition was significantly affected by leaf species when all the data was gathered, however, this significance was only maintained at day 16 when analysing sampling days separately. Lipid body condition and growth rates were not affected by leaf species neither analysing all data together nor at separate sampling days. Results at sampling day 16 were similar to those using all the sampling days (Table 3).

The standard error decreased over time for growth rate and consumption, as predicted (Growth rate, $p < 0.001$; Consumption, $p = 0.0034$, Fig. 3). The standard error of growth rate was significantly reduced from Day 2 (0.019) to Day 4 (0.009), Day 8 (0.004), and Day 32 (0.001). This reduction was statistically significant from day 2 to 16 for consumption, which was reduced from 0.072 to 0.025 and then levelled off until day 32 (Fig. 3). The rest of the measured variables did not show any reduction of the standard error.

Table 2. ANOVA results for measured response variables in *E. berilloni*. The effect of leaf species, experiment duration and interaction between both sources of variation was tested.

Variable	Source of variation	DF	*F-value*	*p-value*
Consumption	Species	4	62.76	<0.001
	Day	4	8.46	<0.001
	Species:Day	16	1.83	0.024
Death rate	Species	4	1.42	0.242
	Day	4	0.48	0.491
	Species:Day	16	0.57	0.682
RNA:BM	Species	4	1.15	0.335
	Day	4	1.12	0.349
	Species:Day	16	1.72	0.044
Growth rate	Species	4	1.52	0.195
	Day	4	3.42	0.009
	Species:Day	16	0.67	0.820
Mass body condition	Species	4	3.57	0.007
	Day	4	4.24	0.002
	Species:Day	16	1.99	0.015
Lipid body condition	Species	4	2.12	0.079
	Day	4	0.55	0.698
	Species:Day	16	1.01	0.442

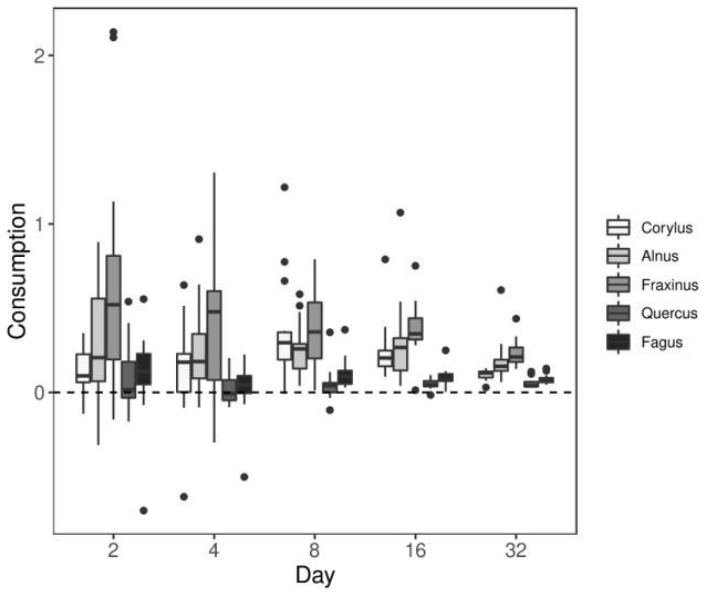


Fig. 1. Consumption rate (mg AFDM · mg BM^{-1} · d^{-1}) of the 5 leaf species versus sampling time. Leaf species ordered by leaf quality from light to dark. The median is represented with the interquartile range (IQR). The upper/lower whisker extends to the largest/lowest value no further than 1.5 * IQR from the hinge. Outliers are represented individually.

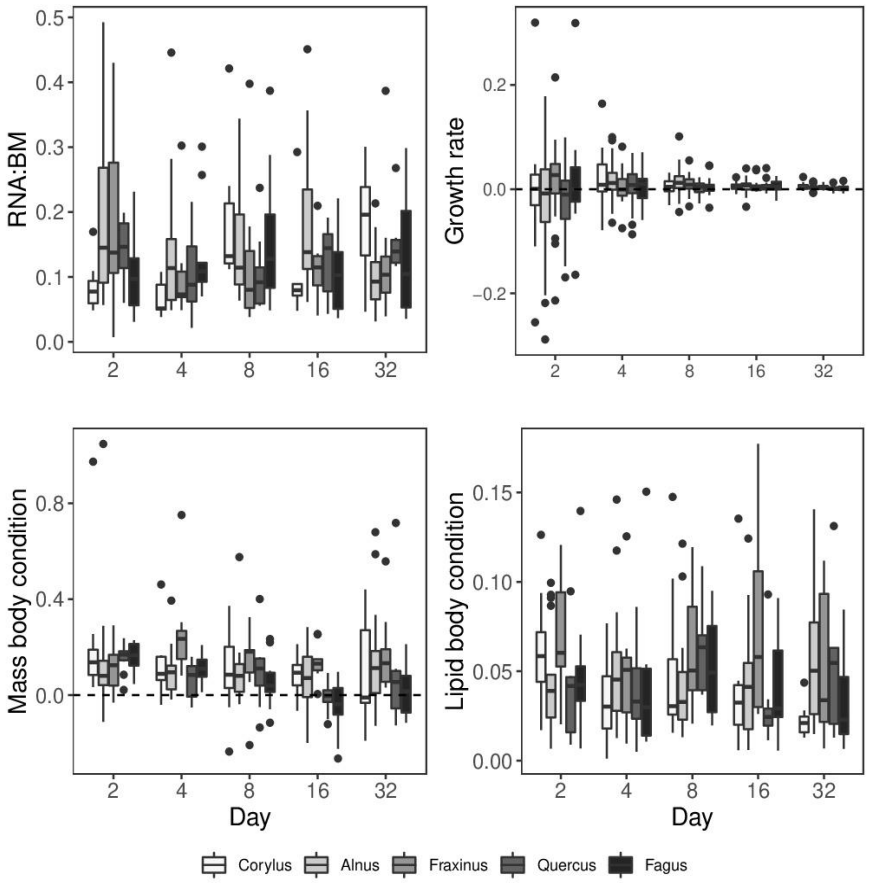


Fig.2. Consumer performance over time feeding on different leaf species. Growth rate (mm · d^{-1}), Mass body condition (log Obs/Exp), Lipid body condition (Lipid · mg BM^{-1}) and RNA:BM (µg RNA · mg BM^{-1}). Leaf species ordered by leaf quality from light grey to dark grey. The median is represented with the interquartile range (IQR). The upper/lower whisker extends to the largest/lowest value no further than 1.5 * IQR from the hinge. Outliers are represented individually.

4. Discussion

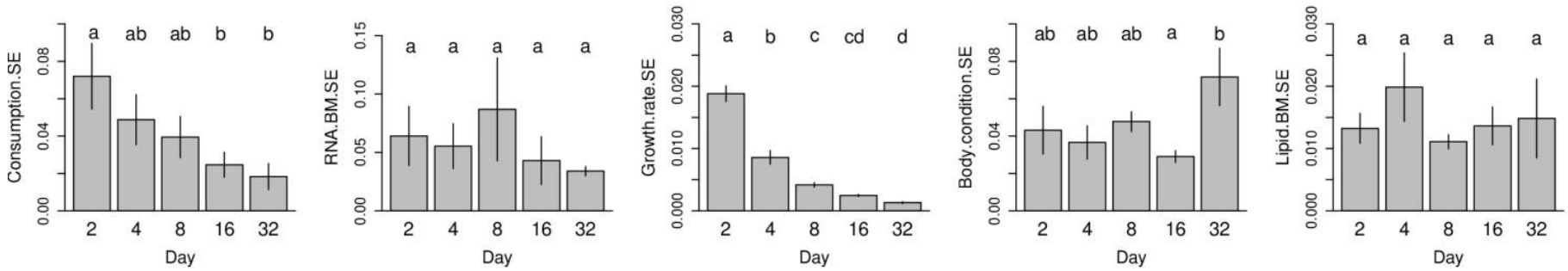
Organic matter consumption is widely measured in the scientific literature due to its importance to support terrestrial and aquatic food webs and the significant contribution to the total carbon fluxes (Battin *et al.*, 2009). Rates of organic matter processing are tightly related with litter quality (Cruz-Rivera & Hay, 2000; Hladyz *et al.*, 2009; Schindler & Gessner, 2009). Our results showed that resource quality affects consumption rates. However, exceptions to the general pattern were also evident, as *Fraxinus* was the most consumed leaf species despite not being identified as the highest quality resource by our quality index. Similarly, in the literature, the correlation between food quality and consumption rates is not always straightforward. Friberg and Jacobsen (1994) observed the feeding preference of *Sericostoma personatum* and *Gammarus pulex* to be unrelated with food source fibre content, toughness, total phosphorus, C:N ratio and total nitrogen content in a 24 h laboratory feeding trial. It has been documented that decomposition rates are lower in substrata with high levels of lignin (Schindler & Gessner, 2009; Ferreira *et al.*, 2016) and tannins (Coq *et al.*, 2010). Even if we did not measure the concentrations of those compounds on our leaf species, Boyero et al. (2017) reported that *Fraxinus* (1.01) has the lowest concentration of condensed tannins of our five leaf species, followed by *Alnus* (1.71), *Corylus* (2.3), *Quercus* (6.23) and *Fagus* (7.07). This points out that the litter quality variables we have used here are not the only drivers for consumption and that other litter traits such as the content of toxic compounds are needed to rank leaf litter in terms of quality. However, resource quality can vary depending on the consumer, as they can be limited by different factors. Santonja et al. (2018), in a 3 d-long laboratory experiment, showed that some invertebrate species (the amphipods *Crangonyx pseudogracilis* and *Gammarus tigrinus* and the caddisfly *Halesus radiatus*) consumed *Alnus* and *Quercus* leaves at similar rates whereas other macroinvertebrate species consumed six times more *Alnus* than *Quercus*. Additionally, they also observed that some invertebrates (the amphipods *C. pseudogracilis* and *E. berilloni* and the caddisfly *S. personatum*) consumed similar quantities of conditioned and unconditioned leaves. Our results relating quality of the resource and its consumption can be, therefore, be included within the variability observed in the literature. However, proving that relationship between resource quality and consumption rate was not the main goal of the present experiment, but to study the consistency of the response along the incubation time on the measured variables.

Even if the quality index did not consider all the leaf quality descriptors, it clearly showed that amphipods were fed with resources significantly contrasting in their quality (i.e., *Corylus*, *Alnus* and *Fraxinus* could be considered high quality resources, while *Quercus* and *Fagus* low quality resources). Those differences in leaf quality were expected to significantly affect amphipod performance based on literature however, most of the measured variables were not significantly affected. For example, it has been demonstrated that nutrient content can limit consumer growth rates (Mulder & Elser, 2009; Kendrick & Benstead, 2013); however, the present resource quality gradient did not to affect individual growth rates. Similarly, in a 6-week feeding trial performed by Graça et al. (2001) the leaf species offered (*A. glutinosa* and *Hura crepitans*), with N:P ratios of 81.21 (calculated from our own data) and 7.6 (from Rincón and Martínez 2006) respectively, did not affect the growth rates of *G. pulex*, *S. vittatum*, *N. argentata* and *P. priapulus*. Regarding amphipod

Table 3. ANOVA results for leaf species and Day as block factor (only when analysing all sampling days) for consumption and amphipod performance. The effect of leaf species is shown for all sampling days and for separate sampling days.

	All samplings				**Day 2**		**Day 4**		**Day 8**		**Day 16**		**Day 32**	
	Day		Species											
	F-value	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*
Consumption	8.03	<0.001	60.86	<0.001	7.92	<0.001	10.67	<0.001	9.53	<0.001	21.89	<0.001	16.85	<0.001
RNA:BM	1.10	0.358	1.37	0.246	2.28	0.075	1.78	0.150	0.84	0.509	2.16	0.090	2.45	0.062
Growth rate	3.45	0.008	1.53	0.193	0.88	0.480	0.61	0.655	1.69	0.159	0.08	0.989	1.18	0.326
Mass body condition	3.72	0.006	3.62	0.007	1.95	0.115	2.48	0.055	0.44	0.776	5.14	0.001	0.71	0.589
Lipid body condition	0.55	0.697	2.14	0.076	1.31	0.278	0.69	0.604	0.16	0.957	1.15	0.345	1.85	0.138

Figure 3. Mean standard errors of consumption and growth rate for the 5 leaf species along time (mean ± SE). Similar letters define groups that do not differ significantly (Tukey´s test, p < 0.01).



physiological status, we did not observe significant responses in RNA:BM ratio of individuals fed with different leaf species. The increase in RNA concentration has been interpreted as a proxy of growth rate (Dahl *et al.*, 2006) and also as a sign of activation of the defence system and repair processes (Maltby, 1999). However, contrary to expected, the RNA:BM ratio did not respond to the resource stoichiometry in the present experiment. Mass body condition, which is directly linked to survival rates (Peig & Green, 2009) and correlates with body protein content (Smock, 1980; Larrañaga *et al.*, 2010), has been also described to respond to resource quality (Larrañaga *et al.*, 2014). In the present study, leaf species significantly affected the mass body condition, individuals fed with high resource quality (*Corylus*, *Alnus* and *Fraxinus*) presenting overall higher mass body condition. Similarly, resource availability and quality have been reported to affect total lipid body condition (Gee, 1988; Øie & Olsen, 1997) and, therefore, have consequences on growth and reproduction (Glazier, 2000); nonetheless, lipid body condition in our amphipods was not significantly affected by the resource gradient. The lack of the response could be related to the low percentage and the high inter-individual variability of the total lipid mass. In line with this, Larrañaga et al. (2014) did not found a significant response to resource quality for the amount of lipids after 3 and 6 weeks of incubation with resources varying in quality as much as in our study.

We predicted that consumption rate and RNA:BM ratio will act as early warning signals and will precede responses occurring later in time, such as changes in growth or body condition. In our experiment consumption rates significantly responded to the resource quality by the second sampling day and the response was consistent over the experiment. However, the measure of the response always depends on incubation duration and the tested impact/factor severity (Haber, 1924). Canhoto and Graça (1996) observed in a 3 months field experiment that differences in consumption rate of eucalyptus, alder, chestnut and oak increased over time. Apart from resource quality, consumption rates are also affected by exposure to toxic compounds. For example, Forrow and Maltby (2000) observed that consumption of *Gammarus pulex* was significantly lower in animals deployed downstream the motorway runoff discharge comparing to the upstream on a 6-d incubation. However, when offered the alternative, individuals did not discriminate between leaf materials that had been previously deployed at both field stations up to 13 days, but after 27 days consumption of upstream-incubated material was significantly higher. The use of consumption rate as early warning signal when testing the effect of consumer-resource stoichiometric imbalance can be quite straightforward because individual feeding preferences/decisions can partially affect their performance. However, when testing other stressors, such as exposure to contaminants, other physiological variables might respond earlier or at the same time that consumption rate. For example, RNA:BM has been shown to rapidly respond to both, pollutant exposure and food quality. Vrede et al. (2002) observed that the RNA:DNA ratio of daphnids responded within 5 hours to differences in food quality and that the RNA:DNA was positively correlated with the C:P ratio. The expected changes in RNA:BM ratio within the first incubation days was not observed in our individuals. Therefore, the RNA:BM cannot be used as early detection tool in this particular case. Finally, it was expected consumption and RNA:BM to precede later responses in growth rate and body condition (i.e., mass body condition and lipid body condition) that would occur as a consequence of different quality resource

consumption. However, growth rate and lipid condition did not respond when taking into account separate sampling days. When analysing sampling days separately, growth rates were not significantly affected by resource type whereas the response was significant taking all the samples together. Individual growth rates were calculated from measurements of the first thoracic segment length at the beginning and the end of the experiment. This measurement has a rather large error that together with the relatively small sample size (n = 20 individuals per leaf species and sampling day), could have avoided to observe significant responses even if there were clear nonsignificant patterns. Leaf species, incubation time and the interaction between both sources of variation significantly affected amphipod mass body condition taking into account all sampling days together. However, when separating sampling days, mass body condition was significantly affected by leaf type at sampling day 16 and the response was disappeared by day 32. Those inconsistencies in the response might be avoided by increasing the sample size.

We have observed incubation time to be an important factor affecting the response. The reduction on consumption rates over time has been attributed to reduced energy requirements under laboratory conditions (Hessen *et al.*, 2013). Additionally, although the main nerves of the leaves were avoided when punching the discs, it is possible that individuals have first eaten the highest quality parts, thus reducing the overall quality and decreasing consumption of the leftovers. Most importantly, for consumption, RNA:BM and mass body condition incubation time significantly interacted with leaf type. The significant interaction between leaf species and incubation time suggest that there are inconsistencies in the response, differences between treatments change over time. The same pattern was observed by Aßmann et al. (2011), who performed a feeding trial (leaves incubated on littoral water or tap water and autoclaved) with *Gammarus roeseli*. During their experiment the preference of *G. roeseli* shifted from autoclaved to littoral-exposed leaves. They attributed the shift on preference to changes on biochemical properties of the leaves over time. Regarding amphipod physiological responses, the interaction between leaf species and incubation duration significantly affected mass body condition and RNA:BM ratio. Similarly, Stuck et al. (1996) found that RNA and protein concentration were not consistent over time on starving *Penaeus vannamei* individuals. Protein concentration initially decreased from day 0 to day 2, then increased at day 4 and gradually decreased again over the experiment. Those variation in response to starvation, and therefore nutritional condition, have been also reported for other crustaceans (Laitinen & Valtonen, 1995; Wu & Dong, 2002; Comoglio, Smolko & Amin, 2005), and might reflect switches in the use of one energy from some types of reserves to another. The observed significant interactions showed the intrinsic difficulty when comparing studies performed with different incubation duration and highlight the importance of time-standardized experiments.

The high variability on the responses observed at the beginning of experiments could be a consequence of the elevated intraspecific variability present in natural populations. The main food source for stream communities, allochthonous organic matter, is distributed in patches in the field (Webster *et al.*, 2001) because different microhabitats differ greatly in the quality of the material retained (Flores *et al.*, 2013). For our study, the amphipods were kick-sampled at different microhabitats along the reach. As a consequence, collected individuals may have presented very

diverse nutritional requirements. Additionally, individual life stage also affects macroinvertebrate body condition and nutritional status (McCahon & Pascoe, 1988), which can explain the large intra-specific variability in stoichiometry that can be observed within a species (Small & Pringle, 2010). We narrowed down the size of the animals used in the experiment, but still the largest individuals were 3 times longer than the smallest ones in our study, which should have contributed to the large variability recorded. Thus, the high variability present in the life stage and the physiological status of the collected amphipods would lead to large variability in their response at the beginning of the experiment, which would later decrease with incubation time. In our study, the predicted reduction in variability was observed for consumption and growth rates. The expected reduction on variability was not observed on mass and lipid body condition and RNA:BM ratio concentration.

Conclusions

Stress signals that are detected on the physiology of biota when facing a perturbation are expected to be followed by changes at the community level and ecosystem functioning. Our results show that both resource quality and incubation duration are relevant factors affecting *E. berilloni* consumption rates, but not for individual performance variables, which showed subtle responses. The interaction between resource quality and incubation time was significant for some of the measured variables, which shows the difficulty when comparing studies performed at different incubatiton times and highlight the need of time standardized experiments. The RNA:BM ratio was no successful as an early warning signal for *E. berilloni*, as it did not show significant differences among the individuals feeding resources of different quality. Although the consumption showed a fast and significant response to resource quality, we could not validate its use as early warning signal because it was not followed by changes in the amphipod performance during the studied time span. Our results, using individuals collected in the field, suggests that studies of at least 16 days of incubation are more reliable as growth rate delivered significant responses and the internal variability was as low as after 32 days of incubation.

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Chapter 2

The presence of alder stimulates the consumption of low quality leaves and affects shredder body condition

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Abstract

The riparian vegetation is being substituted by tree monocultures or agricultural areas worldwide, with detrimental consequences for freshwater systems, very dependent on leaf litter inputs. It has been demonstrated that processing rates of these litter mixtures show non-additive responses, high quality leaf litter stimulating processing of poor resources. However, little is known about the performance of consumers facing a diet based on a litter mixture. Here, we assess how a relatively small availability of high quality resource affects the consumption and performance of the amphipod *Echinogammarus berilloni*. We performed a microcosm experiment where resources of varying quality (*Corylus avellana*, *Fraxinus excelsior*, *Quercus robur* and *Fagus sylvatica*) were offered individually and with the addition of nitrogen-rich *Alnus glutinosa* leaf litter. We predicted that, i) the availability of alder would stimulate the consumption of the predominant material, relatively more when the quality of the resource was low as for *Q. robur* and *F. sylvatica*, and ii) consumer performance would increase in mixtures as complementarity between resources would be beneficial. Consumption of different leaf species significantly differed when offered separately; *A. glutinosa*, *C. avellana* and *F. excelsior* being the most consumed ones. The availability of alder only stimulated the consumption of the worst quality resources (*Q. robur* and *F. sylvatica*), indicating that dissimilarities in quality are the main forces accelerating the consumption rate. With the offered alder amount (20 % of the total surface), a slight increase in growth was observed but body condition and RNA:BM were reduced. The observed reduction in body condition was linked to the quality of the predominant leaf species, high quality resources (*C. avellana* and *F. excelsior*) showing the largest reductions. This reduction might to be related to the metabolic cost of actively searching and comparing resources. Although, contrary to our prediction, amphipod performance did not seem to increase with the availability of alder, we predict an increase of the quantity and quality of the fine particulate organic matter, which would be related to a larger secondary production of other consumers in natural systems.

Keywords: Organic matter quality, non-additive responses, effect size, consumption, amphipod performance

1. Introduction

In headwater streams, where light is limited by natural riparian forest, leaf litter is the main source of energy and nutrients for the stream food webs (Webster & Benfield, 1986; Cummins *et al.*, 1989). Plant species from the riparian forest produce litter that can differ greatly in terms of chemical composition, including the carbon to nitrogen ration (C:N) and concentrations of secondary compounds (e.g., lignin, phenols, tannins; Berg & Meentemeyer, 2002). Additionally, tree species present different abscission patterns, leading to different resource quality and quantity along the year (Pozo *et al.*, 1997; Martínez *et al.*, 2016). Land uses are changing throughout the globe and can be considered the main perturbation in many parts of the world competing with climate change for being the main driver for biodiversity decline (Titeux *et al.*, 2016). In particular, riparian vegetation diversity has been decreasing worldwide via substitution of forests with monocultures and degradation of riparian corridors by agricultural and industrial activities (Graça *et al.*, 2002; Aguiar *et al.*, 2007; Van Wilgen, Nel & Rouget, 2007; Kominoski *et al.*, 2013). These changes in land use alter directly the availability and quality of coarse particulate organic matter (CPOM) along streams and rivers and thus, alter energetic linkages between aquatic and terrestrial habitats.

Organic matter incorporated into streams accumulates in the most retentive locations of the streambed creating leaf litter batches of very heterogeneous quality (Flores *et al.*, 2013). Decomposition patterns of those leaf litter mixtures are not always predictable from single-species dynamics (e.g., García et al., 2012). The response has been defined as "additive" when decomposition in litter mixtures are predictable from single species decay rates, whereas "non-additive" responses when it deviates from predicted responses (Gartner & Cardon, 2004). While some authors highlight the importance of species richness in maintaining decomposition rates (LeRoy & Marks, 2006; Kominoski *et al.*, 2007) other author suggest that species identity in a mixture is more important than richness to maintain the breakdown rates (Swan & Palmer, 2006a; Abelho, 2009). Moreover, some experiments have shown that high quality materials are able to promote litter decomposition of poor resources (e.g., Kominoski et al., 2011; Lecerf et al., 2005; McKie and Malmqvist, 2009). For example, the riparian tree species *Alnus glutinosa* is a high quality resource with elevated nitrogen concentration (1.95 % of dry mass) and low concentration of secondary metabolites (tannin concentration 1.71% of dry mass, Boyero *et al.* (2017)), which have been observed to stimulate decomposition rates in leaf litter mixtures (Ferreira et al., 2012). In headwater streams that have not been altered by human activities high quality leaf species, such as *A. glutinosa*, are far from being predominant on stream benthic organic matter (Pozo *et al.* (1997) reported that 20.5 % of the total stock of organic matter was alder), and yet they are very productive and diverse freshwater systems.

Whereas processing rates of litter mixtures have been thoroughly documented (e.g., Ferreira et al., 2012; Schindler and Gessner, 2009; Taylor et al., 1989), the performance of consumers facing a mixture of leaf litter has been much less studied (but see Swan & Palmer, 2006b; Flores, Larrañaga & Elosegi, 2014b; Landeira-Dabarca *et al.*, 2018; López-Rojo *et al.*, 2018). Although consumers can exhibit preferential feeding when foraging on resources of varying palatability (Cruz-Rivera & Hay, 2000; Hladyz *et al.*, 2009; Schindler & Gessner, 2009), litter mixtures

stimulate the consumption of refractory resources (Ylla *et al.*, 2010; García *et al.*, 2012). Consumption rates and consumed leaf litter quality have direct effects on consumer's performance; shredders growth rate (Mulder & Elser, 2009; Kendrick & Benstead, 2013), body condition in terms of mass and lipid condition (Gee, 1988; Øie & Olsen, 1997; Larrañaga *et al.*, 2014) and RNA:BM (Stuck *et al.*, 1996). There is also evidence that consumers can choose to have a mixed diet in order to consume complementary nutrients or to dilute the toxicity of secondary compounds (Freeland & Janzen, 1974; Pennings *et al.*, 1993; Bernays *et al.*, 1994). When resources are complementary, a consumer requires a lower amount of resources when consumed in combination than consumed individually (Tilman, 1982). Resource complementarity was tested by Pennings et al., (1993), who observed that the gastropoda *Dolabella auricularia* grew better when offered a diet consisting on algae mixtures instead of single algae diet. For detritivores, we could expect consumption of litter mixtures to have complementary effect and enhance their performance.

In the present study, we assess how the addition of a relatively small quantity of high-quality leaf litter affects the consumption rate and performance of a detritivore. In a microcosm experiment we reared a detritivore (*Echinogammarus berilloni*) with resources of varying quality (*Corylus avellana*, *Fraxinus excelsior*, *Quercus robur* and *Fagus sylvatica*) individually and with the addition of nitrogen-rich *Alnus glutinosa* leaf litter. We predicted that, i) the availability of alder would stimulate the consumption of the predominant material (non-additive responses), relatively more when the resource quality was lower and ii) consumer performance would increase in mixtures as complementarity between resources will appear.

2. Materials and methods

2.1. Organic matter and taxa selected

Leaf litter of five common tree species in the riparian corridor of European streams were used in the experiment. These species were chosen because they largely differ on their traits, such as nutrient content or toughness (Boyero *et al.*, 2017). *Alnus glutinosa* ((L.) Gaertn), *Corylus avellana* (L.), *Fraxinus excelsior* (L.), *Quercus robur* (L.) and *Fagus sylvatica* (L.) leaves were collected near Bilbao (North Iberian Peninsula 43º15´47´´ N 2º56´06´´ W). Leaves were collected after abscission in October 2015 and disks (12 mm diameter) were extracted using a cork borer, air dried and stored in 24-well culture plates in a dark dry place until further use. Leaf litter elemental analyses were performed for a total of 5 replicates for each species. To characterize the quality of the 5 different resources, leaf disks were ground and homogenized. For nitrogen and carbon content measurements, samples were weighed in tin capsules and analyzed with Eurovector EA 3000 CNH analyzer (Eurovector, Milan Italy). For phosphorus and potassium analysis, the freeze-dried samples were subjected to acid digestions (0.5 g in 15 mL of HNO_3 69 %). For total concentration characterization, optical emission spectrophotometer with inductively coupled plasma (ICP-OES, Horiba Jobin Yvon, Activa) was employed. Leaf toughness was measured using a penetrometer with a 0.49 mm^2 area steel rod (Boyero *et al.*, 2011a) (15 replicates per species).

As consumer macroinvertebrate we chose the amphipod *Echinogammarus berilloni* (Catta.) as it is one of the most abundant detritivore in the streams of the region (Larrañaga et al., 2009) and has been shown to be sensitive to resource quality (Larrañaga et al., 2009; Larrañaga et al.,

2014). Specimens were kick-sampled (500 µm pore size) in the Agüera basin (Adino 43º20´27´´ N 3º20´20´´ W), individually enclosed in 30 mL containers with pores for water interchange and carried to the laboratory immersed in transport boxes filled with stream water. Animals with a first thoracic segment length between 0.24 and 1.26 mm were selected (4.59 and 17.08 mm in total body length), but breeding females were discarded as they show a clearly differentiated biochemical composition (Larrañaga et al., 2009). The animals were acclimatized in a controlled temperature room without food for 3 d prior to the experiment.

2.2. Experimental setup

We prepared a set of microcosms consisting of test tubes (2 cm diameter and 20 cm long, enough to allow free movement of the amphipods) filled with 40 mL of filtered (100 µm) stream water. Two types of microcosms were prepared a) microcosms with a single leaf species and, b) microcosms with two leaf species in which one of them was always alder and the others were referred as predominant disks. In microcosm with a single leaf species 5 disks of one leaf species (i.e., *Corylus*, *Alnus*, *Fraxinus*, *Quercus* and *Fagus*) were offered to the amphipod. In microcosms with two leaf species we added 4 leaf disks of *Corylus*, *Fraxinus*, *Quercus* or *Fagus* and a single leaf disk of *Alnus*. Hereafter, the name of the predominant disk will correspond to the treatment name. Prior to the experiment, the groups of 5 leaf disks were conditioned in stream water at the microcosms for 15 days at 15 ºC, 12:12 light photoperiod and constant aeration (water renewed at day 7). The conditioning was aimed at reducing the leachates and promoting microbial colonization. After conditioning, water was renewed and an individual of *E. berilloni* was added per microcosm. Experimental conditions were kept as during conditioning. The experiment lasted 32 days and water was renewed at days 8, 16 and 24 to avoid accumulation of deleterious substances and shortage of nutrients. In order to analyze the amphipod response over time, microcosms were samples at days 2, 4, 8, 16 and 32 (variables determined below). Replication for each sampling day and leaf species was 20 (20 replicates x 5 treatments x 5 sampling times x 2 microcosm type), in total 1000 microcosms.

2.3. Response variables

The study focused on five variables, one variable related to functioning (organic matter consumption rates) and four variables related to the amphipod performance (growth rate, body mass condition, lipid condition and RNA:Body mass ratio).

2.3.1. Consumption rates

The five air-dried disks of each microcosm were weighed before the conditioning. When two leaf species were offered in the same microcosm, leaf species were weighed separately: the four disks of the predominant species together on one hand and the alder disk on the other hand. At each sampling day, disks were retrieved from the microcosms. Here again, when two leaf species were offered in the same microcosm, leaf species were handled separately. The collected material was oven dried (70 ºC, 72 h) and ashed (500 ºC, 4 h) to obtain the ash free dry mass (AFDM). Mass loss in the conditioning was estimated and used to correct the initial AFDM for leaching and microbial decomposition. An additional set of 9 microcosms per leaf species were incubated without amphipods during the experiment to estimate microbial decomposition rates and therefore correct the consumption rates. Consumption was calculated subtracting final AFDM to the initial AFDM and standardized by dividing

it by the geometric average body mass of the individual and duration (days) of incubation (mg AFDM · mg BM^{-1} · d^{-1}). On microcosm containing two leaf species, total consumption rate and separate consumption of each leaf species were calculated.

2.3.2. Growth rate

Amphipods were photographed at the beginning and end of the experiment with a binocular microscope (Leica M165FC, Wetzlar, Germany). The dorsal length of the first thoracic segment (*DL*) was measured from photographs using the "Leica Application suite V4" program (LAS V4.1). Initial and final total body lengths (*BL*) were calculated using Eq. (1) (Flores *et al.*, 2014a). Instantaneous growth rate (*IGR*) for each individual was calculated using Eq. (2) where *t* is the incubation duration, BL_t is the body length at time *t* and BL_0 is the initial body length (Flores *et al.*, 2014a).

Eq. (1) $BL = 14.458 \cdot DL - 0.110$; (mm)

Eq. (2) $IGR = (\ln(BLt) - \ln(BL_0)) / t$; (mm · d^{-1})

2.3.3. Body condition: mass and lipids

By means of the Eq. (3) (Flores *et al.*, 2014a) the body mass (*BM*) was estimated at the end of the experiment, and this value was used as the descriptor for each invertebrate dry mass for body condition at the end of the experiment.

Eq. (3) $BM = 0.8213 \cdot BL - 4.3025$; (mg)

Half of the sampled amphipods (10 replicates per leaf species, sampling day and microcosm type) were freeze dried and weighed. The weighed dry mass of the individual was divided by the expected body mass calculated from the body length (Eq. 3) and logaritmized to calculate the Body Mass condition. A value of 0 indicates that the weighed mass exactly matches what is expected from the body length, while positive and negative values show heavier or lighter than expected individuals, respectively. Total individual lipid content was measured as a proxy of stored energy reserves. Lipid amount was quantified in freeze-dried entire individuals. Extraction was performed incubating individuals in diethyl ether at 4 ºC for 2 days and sonicated twice for 15 min (Bandelin Sonorex; Bandelin Electronic GmbH and Co., Berlin, Germany). Extraction was followed by digestion with H_2SO_4 (200 ºC) and quantification by spectrophotometer. Cholesterol was used as standard. Lipid condition was expressed as Lipid · mg BM^{-1}.

2.3.4. Nucleic acid content

The rest of the individuals (10 replicates per leaf species, sampling day and microcosm type) were stored in RNAlater solution (Ambion) until further nucleic acid analysis. Microplate fluorometric high-range assay with Ribo-Green was performed to quantify RNA after extraction with *N*-laurylsarcosine followed by RNase digestion as described in detail by Gorokhova and Kyle (2002). Fluorescence measurements were performed using a Tecan GENios microplate reader (Cavro Scientific Instruments, Sunnyvale, filters: 485 nm excitation and 520 nm emission). Measurements were performed in black solid flat-bottom microplates, scanned with 0.2 s · $well^{-1}$ measurement time, with 10 measurements per well at constant temperature (37 ºC). Measured RNA concentrations were expressed as µg RNA · mg BM^{-1}.

2.4. Data analysis

A high degree of correlation was observed among the leaf traits (minimum R^2 in pairwise Pearson correlation: 0.67; see Supplementary material A from Chapter 1). Thus, we opted to integrate the

information of all the variables by computing an index of leaf litter quality. This quality index was calculated by means of Eq. (4), where *T* is the measured value for the leaf trait and *n* the number of measured traits.

Eq. (4) *Quality index* = $1 - ((T_1 / (n \cdot T_{1(max)})) + \ldots + (T_n / (n \cdot T_{n(max)})))$

Death rate was expressed as the natural logarithm (Ln) of the difference between initial and final number of individuals alive per treatment and sampling day, standardized by incubation duration (days).

The *Net effect size* of the availability of alder disk was calculated (following Krebs, 1989) for consumption and amphipod performance based on responses obtained from incubations with single leaf species and two leaf species. This value was calculated by means of Eq. (5) where "$Sp._{+2}$" was the response of amphipods fed with two leaf species, "$Mean.Sp_{.+1}$" was the mean value of the response at single species microcosms and "$SD_{Sp.+2}$" was the standard deviation of the response at microcosms with two leaf species.

Eq. (5) Net Effect size = $(Sp._{+2} - Mean.Sp_{.+1}) / SD_{Sp.+2}$

A value of 0 in Net effect size indicates that the alder disk availability did not affect the response, while positive and negative values show an increase or a reduction of the variable considered created by the availability of the alder disk.

Additionally, the *Relative effect size* of the availability of alder disk was calculated (following Krebs, 1989) based on observed and expected response values. The observed values consist on the measured values for the response variables at the microcosm with two leaf species. The expected values were calculated from microcosm with single leaf species. For those microcosms, a mean value of the response was calculated for each leaf species. This mean value was divided by 5 (the number of leaf disk per microcosm) in order to normalize the response per disk. Finally, the expected value was calculated by adding the response mean value of 4 corresponding predominant disks and one alder disk, as it was the resource availability in the microcosm with two leaf species. The relative effect size was calculated by means of Eq. (6) subtracting the expected mean values (Mean.Exp) to the observed values (Obs) and divided by the standard deviation of the observed values (SD_{Obs}).

Eq. (6) Relative *Effect size* = (Obs – Mean.Exp) / SD_{Obs}

Positive relative effect sizes indicate that the measured value is higher than expected and therefore, that the availability of an alder disk promotes more than expected the consumption or the performance of the consumer. Negative relative effect sizes indicate that the measured value is lower than the expected from the additive combination of the responses in monocultures.

We fitted Gaussian models (Madsen & Thyregod, 2010a; Zuur & Ieno, 2010b) to test differences in leaf disk characteristics. Linear models were fitted, where the effect of Species (fitted as discrete explanatory variable) was tested on leaf stoichiometry (C:N, C:P, C:K ratios) and toughness. To test the effect of leaf species on consumption, linear mixed-effect models were used, via the lmer() function of the package nlme (Pinheiro *et al.*, 2018). The fixed structure of the models included leaf species (fitted as a discrete explanatory variable, with five levels, *Alnus*, *Corylus*, *Fraxinus*, *Quercus* and *Fagus*) and incubation time (days) as the random factor. Additionally, in order to test for the effect of alder disk availability to predominant leaf species disk both effect sizes (Relative

and Net) were calculated for all the response variables, which were total consumption rate, predominant disks consumption rate, alder disk consumption rate, amphipod growth rate, body mass condition and body lipid condition and RNA:BM ratio. For parameter estimation, linear mixed-effect models were used, via the lmer() function of the package nlme (Pinheiro *et al.*, 2018). The fixed structure of the model included the predominant leaf species (fitted as a discrete explanatory variable, with four levels, *Corylus*, *Fraxinus*, *Quercus*, and *Fagus*) and incubation duration (days) as random factor. No heteroscedascity was observed among the different leaf species. When needed data was normalized via the gaussianize() function of the package LambertW (Goerg, 2011). Turkey's HSD tests were carried out for post-hoc pairwise comparisons between different leaf species. The critical alpha value for all analyses was 0.05 and all statistical analyses were conducted using R statistical software (version 3.1.2, R Core Team, 2018).

3. Results

Stream water used over the experiment ranged in phosphorus concentration from 4.7 $\mu g \cdot L^{-1}$ to 28.2 $\mu g \cdot L^{-1}$ and in ammonia concentration from 35.6 $\mu g \cdot L^{-1}$ and 106.7 $\mu g \cdot L^{-1}$. The means values of Cl, SO_4 and NO_3 were 11.4 $mg \cdot L^{-1}$, 7.9 $mg \cdot L^{-1}$ and 2.4 $mg \cdot L^{-1}$ respectively.

The four predominant leaf litter species used in the experiment differed from alder in stoichiometry (C:N, C:P, C:K ratios) and toughness and consequently, in the quality index (Table 1). *Corylus* showed higher quality index than alder, due to its high phosphorus and potassium concentration and softness, however the former present lower nitrogen concentration (Table 1). *Fraxinus* have lower nitrogen concentration than alder but they did not differ in the rest of the measured variables (Table 1). The two species that most differed from alder in terms of quality were *Quercus* and *Fagus*, presenting significantly lower nutrient concentration and higher toughness (Table 1).

Consumption of the resources significantly differed when offered separately ($p < 0.001$, Table 2, Fig. 1). The highest quality leaves (*Alnus*, *Corylus* and *Fraxinus*) were the most consumed ones, significantly more than *Quercus* and *Fagus*. The presence of the alder disk affected differently the consumption depending on the quality of the predominant leaf species ($p < 0.001$, Table 2, Fig. 2). Alder availability had a stimulating effect on total consumption when offered with *Quercus* and *Fagus* (i.e. total consumption rate was higher with alder than in cultures with a single resource; Fig. 2A). This stimulation was related to a higher than expected consumption of both the predominant resource and alder (Fig. 2C and 2D). When alder was offered together with other high-quality resources, *Corylus* and *Fraxinus*, consumption rates showed an additive response. In this case, both total consumption and the consumption of the predominant species in mixture cultures was not significantly different to the amount anticipated from monocultures (i.e. effect size = 0) (Fig. 2). On the other hand, with *Corylus*, alder consumption was stimulated whereas this did not happen with *Fraxinus* (Fig. 2).

Table 1. Sumary of the characteristics of the materials used in the experiment. Nitrogen, phosphorus and potassium to carbon ratios (n = 5) and leaf toughness (kPa) (n = 15) values are shown. Mean values and standard error are presented. Non-matching letters indicate statistically different groups (Tukey´s test, $p < 0.05$).

Leaf litter sp.		
C:N		
	Alnus	$14.05 \pm 0{,}07^{a}$
	Corylus	19.92 ± 0.38^{b}
	Fraxinus	22.76 ± 0.66^{c}
	Quercus	37.39 ± 0.40^{d}
	Fagus	52.70 ± 0.11^{e}
C:P		
	Alnus	1141.87 ± 20.65^{a}
	Corylus	838.69 ± 11.20^{b}
	Fraxinus	1128.87 ± 31.03^{a}
	Quercus	3259.20 ± 84.56^{c}
	Fagus	2073.13 ± 69.22^{d}
C:K		
	Alnus	1920.94 ± 97.92^{a}
	Corylus	1157.70 ± 75.88^{b}
	Fraxinus	1719.30 ± 143.15^{a}
	Quercus	2804.83 ± 75.40^{c}
	Fagus	2729.15 ± 66.74^{c}
Toughness (kPa)		
	Alnus	70.47 ± 4.96^{ab}
	Corylus	57.81 ± 3.09^{a}
	Fraxinus	81.52 ± 6.51^{b}
	Quercus	123.48 ± 8.53^{c}
	Fagus	$107.4\ 3\pm 5.04^{c}$
Quality index	Alnus	0.532
	Corylus	0.621
	Fraxinus	0.487
	Quercus	0.130
	Fagus	0.073

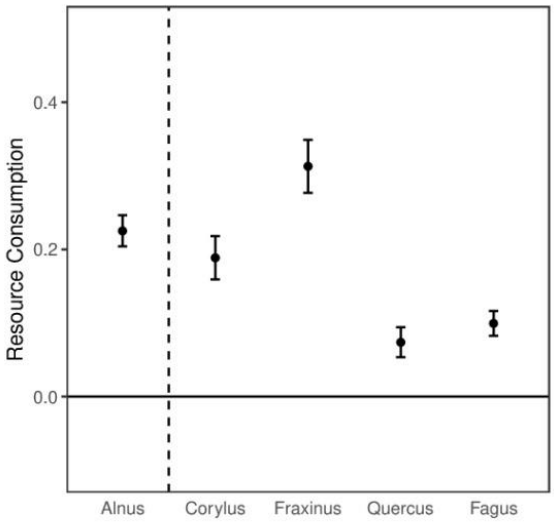

Figure 1. Resource consumption in monocultures (mg AFDM · mg BM^{-1} · d^{-1}). Mean value and confidence intervals are represented for each leaf species.

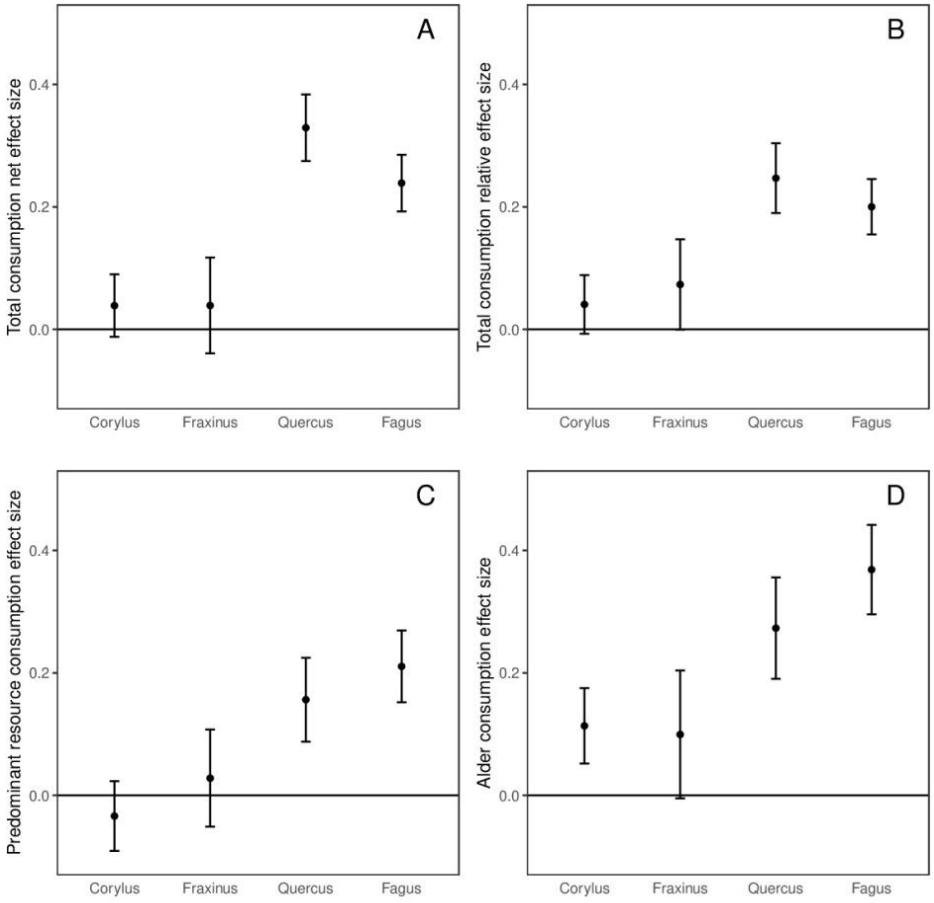

Figure 2. A) total consumption (predominant + alder disk) net effect size, B) total consumption (predominant + alder disk) relative effect size, C) predominant disk consumption relative effect size and D) alder disk consumption relative effect size. Mean value and confidence intervals are represented for each leaf species; thus, effect size intervals that do not cross Y = 0 can be considered significantly different from the monoculture (Net effect size) or the expected (Relative effect size) outcome.

The availability of the alder disk significantly stimulated growth rates when *Corylus* or *Quercus* was the predominant species, although the average growth rate tended to increase in all cases (Net effect size, Fig. 3). The increment of growth was not significantly different to what we estimated from single species incubations, i.e. the relative effect size of alder availability was additive, for all but for *Quercus*, in which we recorded higher than expected growth rates (Fig. 3). Neither net nor relative effect sizes of growth rate differ statistically among the different predominant resources (Table 3, Fig. 3).

The availability of the alder disk together with *Corylus*, *Quercus* and *Fagus* did not significantly change the mass body condition of amphipods, while the availability of alder disk together with *Fraxinus* disks significantly decreased the mass body condition of individuals (Net effect size, Fig. 3). Individuals fed with *Corylus* and alder mixture present worst mass body condition than expected (Relative effect size, Fig. 3). The effect of alder disk availability on the net and relative effect sizes of amphipod body mass condition was related to the quality of the resource, with highest quality predominant resources reducing more the body condition with the availability of alder (Table 3, Fig. 3). Individuals fed with predominant disks mixed with alder showed lower lipid body condition than individuals fed with only predominant disk (Net effect size, Fig. 3). Additionally, individuals from all the treatments present worst body lipid condition than expected (Relative effect size, Fig. 3). As well as for mass condition, both effect sizes of alder disk availability on amphipod body lipid condition were significantly affected by resource (Table 3, Fig.3), and again, high quality predominant species showing the largest reduction of the lipid condition. Individuals fed with *Corylus* and *Fraxinus* disks enriched with an alder disk present lower RNA:BM values than individual fed with only predominant disk. Additionally, amphipods from *Corylus* and *Fraxinus* treatments present lower RNA:BM than expected while individuals from *Quercus* and *Fagus* treatments showed additive responses (Relative effect size Fig. 3). The amphipod RNA:BM ratio relative and net effect size responses were significantly affected by leaf species (Table 3, Fig.3).

4. Discussion

Mixing leaf litter of different quality can promote the overall consumption rates in terrestrial and aquatic ecosystems through the process denominated "priming effect" (Guenet *et al.*, 2010). Tonin et al., (2017) provided evidence that leaf litter diversity increases decomposition rates through complementarity effects, which are mediated by both, microbial and detritivore macroinvertebrate assemblages. During a field experiment Taylor et al., (1989) also observed a marked acceleration in mixed litter microbial decomposition in leaf litter bags that were filled with both *Populus tremuloides* and *Alnus crispa* compared to bags filled with only one of the two species. Similarly, when different quality leaves were incubated in a stream-side channel by García et al., (2012), litter consumption by the invertebrate community was faster in mixtures than in single species incubation, suggesting that rapidly decaying litter stimulates consumption of adjacent and more recalcitrant leaves. Accordingly, in the present experiment, we observed that the availability of a high-quality leaf litter (i.e., *Alnus* disk), had a non-additive stimulating effect on the total consumption rate when offered together with low quality *Quercus* and *Fagus* leaf litter. Consumers have been seen to maintain mixed diets in order to ensure the acquisition of various nutrients simultaneously (Rapport, 1971; Pulliam, 1974; Westoby, 1978).

Table 2. ANOVA results for the single resource consumption and effect size of the availability of alder on, total consumption (net and relative) and, predominant disk and alder disk consumption rates (mg AFDM · mg BM^{-1} · d^{-1}). The effect of leaf species was tested. Non-matching letters indicate statistically different groups (Tukey´s test, $p < 0.05$).

Variable	**numDF**	**denDF**	***F-value***	***p-value***	**Tukey**				
					Alnus	*Corylus*	*Fraxinus*	*Quercus*	*Fagus*
Single resource consumption	4	533.12	51.23	<0.001	a	a	b	c	c
Total consumption with alder net effect size	3	348.27	33.73	<0.001	-	a	a	c	b
Total consumption with alder relative effect size	3	351.33	19.88	<0.001	-	a	a	b	b
Predominant disk consumption relative effect size	3	351.37	15.46	<0.001	-	a	a	b	b
Alder disk consumption relative effect size	3	351.47	12.87	<0.001	-	a	a	b	b

Table 3. ANOVA results for the Net and Relative effect size of alder availability on amphipod performance. The effect of leaf species was tested. Non-matching letters indicate statistically different groups (Tukey´s test, $p < 0.05$).

Variable	**Effect size**	**DF**	***F-value***	***p-value***	**Tukey**			
					Corylus	*Fraxinus*	*Quercus*	*Fagus*
Growth rate	Net	3	1.76	0.155				
	Relative	3	0.46	0.709				
Mass body cond.	Net	3	1.04	0.374				
	Relative	3	2.76	0.044	a	ab	ab	b
Lipid body cond.	Net	3	35.71	< 0.001	a	bc	b	c
	Relative	3	4.26	0.006	a	ab	ab	b
RNA:BM	Net	3	13.19	< 0.001	a	a	b	b
	Relative	3	2.73	0.046	a	ab	b	ab

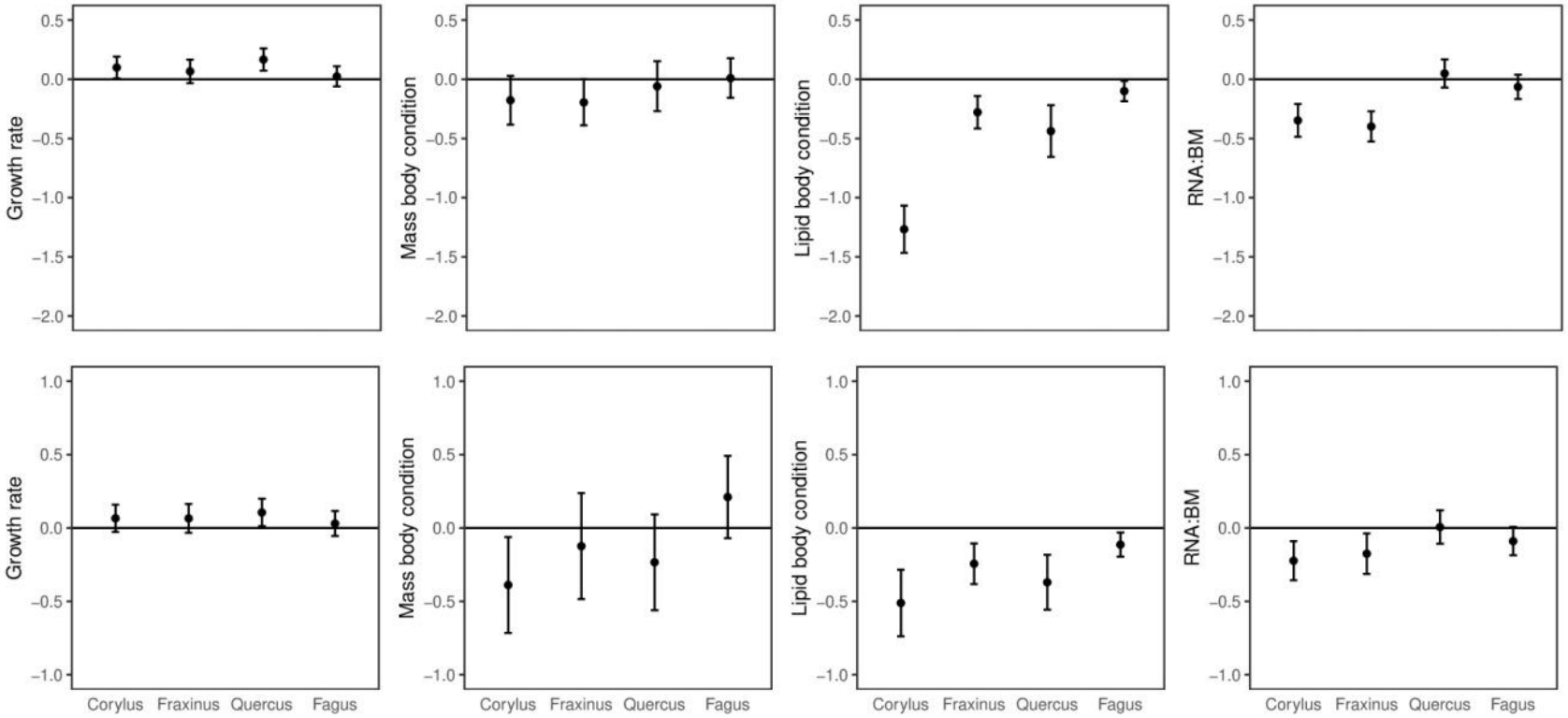

Figure 3. Effect of the availability of the alder disk on the performance of the amphipod measured in growth rate, body mass condition, body lipid condition and RNA:BM ratio. Net effect size of alder availability (above) and Relative effect size of alder availability (below). Mean value and confidence intervals are represented for each leaf species; for effect sizes intervals that do not cross Y = 0 can be considered significantly different from the monoculture (Net effect size) or the expected (Relative effect size) outcome.

Apart from getting a wider range of resources, Freeland and Janzen, (1974) suggested that having diversified diets could help avoiding consuming too much of any toxic compound. As a mechanism for this stimulating effect, García et al. (2012) pointed out that nitrogen and phosphorus losses in alder where faster in litter mixtures than when incubated alone. Those nutrients released to the medium could stimulate the growth of the microbial community and therefore, palatability of the most recalcitrant leaf species increasing their consumption (Kuzyakov, Friedel & Stahr, 2000; Guenet *et al.*, 2010). In the present experiment, when the predominant leaf litter was also of high quality, i.e. with *Corylus* and *Fraxinus* we observed an additive effect of alder availability on consumption, whereas when the predominant resource was of low-quality (i.e. *Fagus* or *Quercus*) the stimulating effect appeared. Similarly, Santschi et al., (2018) performed a laboratory experiment where microbial decomposition was measured in leaf species alone and in mixtures. They found non-additive stimulating effects when mixing *Fagus* or *Quercus* (low quality) with alder (high quality) indicating that dissimilarities in resource quality were the main force accelerating the consumption rates.

Changes in consumption rates and ingested resource quality are expected to have consequences on consumer's performance. In litter mixtures, each resource can provide different nutrients and although detritivore consumers can survive feeding only one resource (Graça *et al.*, 2001; Carvalho & Graça, 2007), they increase their performance on some mixtures (Westoby, 1978). In the present experiment, growth rates increased in *Corylus* and *Quercus* treatments as a consequence of the availability of alder and even if it was not statistically significant, individuals from *Fraxinus* and *Fagus* treatments drifted towards having higher growth rates when alder was added. Moreover, the growth rate at *Quercus-Alnus* mixture was non-additive, showing a higher growth rate than expected from values recorded in single species incubations. However, enhanced consumption in litter mixtures is not always translated into higher growth rates (Frainer *et al.*, 2016). Higher feeding rates brings more food into the gut and reduces the gut transit time, thus reducing the assimilation efficiency (Levinton, 2013). In our experiment, the reduction on assimilation efficiency derived from higher consumption rates could be linked to the reduced body condition and RNA:BM ratios. This result was contrary to what was expected, but very consistent as the body condition and the RNA:BM ratio did not increase with the alder availability at any of the treatments. With the amount of alder offered to the amphipods we seem to have triggered a slight increase in longitudinal growth rate, but not a proportional gain in mass, which can partly explain the reduction of the body condition. Interestingly, the reduction of body condition was also related to the quality of the predominant resource, with high-quality *Corylus* and *Fraxinus* showing the largest reduction on condition when alder was added. This differential change in the body condition among resources cannot be explained with the growth rate (individuals growing more longitudinally would show the largest reductions in body condition and there were no significant differences for the growth rate among different treatments). A tentative explanation is that resource mixtures increase the metabolic cost of the consumer that needs to search and compare more actively the offered resources. With the non-additive stimulating consumption that we observed for low quality resources (*Fagus* and *Quercus*) when alder was added the extra metabolic cost could have been compensated with the larger consumption rate. For the high-quality resources

(*Corylus* and *Fraxinus*) alder availability did not trigger the stimulation of the consumption, and thus, the extra metabolic cost related to resource acquisition could not be compensated. This explanation requires further experiments: one with a range of alder availability might shed some light on the matter, as a higher availability of the nutrient-rich material would compensate for the metabolic cost observed.

Total secondary production of an ecosystem comes from a variety of consumers that acquire their resources in very different ways. In streams the total diversity, abundance and biomass of consumers that feed on smaller particles, filtering from the water column or collecting them from the benthos, is larger than for shredders (Cummins & Klug, 1979). Nevertheless, shredders are responsible for the fragmentation of CPOM into smaller ones through their feeding activity and the production of faeces, which leads to the production of fine particulate organic matter (FPOM) (Allan & Castillo, 2007). Thus, it is assumed that leaf processing by shredders through the "processing chain" enhances the resource availability and production of collectors, although direct evidence is rare (Heard & Richardson, 1995). More clearly demonstrated is the fact that the amount of FPOM has a positive correlation with invertebrate densities (Callisto & Graça, 2013), as it is an important driving factor behind the habitat choices of collectors (Brady & Cowell, 2003). This way, FPOM production and downstream transport are considered key processes on conceptual models of stream ecosystem structure and functioning, such as River Continuum Concept (Vannote *et al.*, 1980). In our study we observed that the availability of alder stimulated the processing rates of leaf litter, which should have produced higher amounts of FPOM as was observed in other studies (Cuffney, Wallace & Lugthart, 1990; Ferreira *et al.*, 2013). The alder availability should had therefore increase both, quantity and quality of FPOM, which, in a natural system, would have been translated into a larger secondary production of filterers and collectors, with positive consequences for higher trophic levels such as predator invertebrates or fish (Shepard & Minshall, 1984a b).

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Chapter 3

Testing wastewater treatment plant effluent effects on microbial and detritivore performance: a combined field and laboratory experiment

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Abstract

The amount of pollutants and nutrients entering rivers via point sources is increasing along with human population and activity. Although wastewater treatment plants (WWTPs) greatly reduce pollutant loads into the environment, excess nutrient loading is a problem in many streams. Using a Community and Ecosystem Function (CEF) approach, we quantified the effects of WWTP effluent on the performance of microbes and detritivores associated to organic matter decomposition, a key ecosystem process. We measured organic matter breakdown rates, respiration rates and exo-enzymatic activities of aquatic microbes. We also measured food consumption and growth rates and RNA to body-mass ratios (RNA:BM) of a dominant amphipod *Echinogammarus berilloni*. We predicted responses to follow a subsidy-stress pattern and differences between treatments to increase over time. To examine temporal effects of effluent, we performed a laboratory microcosm experiment under a range of effluent concentrations (0, 20, 40, 60, 80 and 100 %), taking samples over time (days 8, 15 and 30). This experiment was combined with a field *in situ* Before-After Control-Impact Paired (BACIP) experiment whereby we added WWTP effluent poured (10 L s^{-1} during 20-40 min every 2 h) into a stream and collected microbial and detritivore samples at days 8 and 15 (5 and 15 replicates to assess the microbe and detritivore performance respectively, per period, reach and sampling day). Responses were clearer in the laboratory experiment, where the effluent caused a general subsidy response. Field measures did not show any significant response, probably because of the high dilution of the effluent in stream water (average of 1.6 %). None of the measured variables in any of the experiments followed the predicted subsidy-stress response. Microbial breakdown, respiration rates, exo-enzymatic activities and invertebrate RNA:BM increased with effluent concentrations. Differences in microbial respiration and exo-enzymatic activities among effluent treatments increased with incubation time, whereas microbial breakdown rates and RNA:BM were consistent over time. At the end of the laboratory experiment, microbial respiration rates increased 156 % and RN:BM 115 % at 100 % effluent concentration. Detritivore consumption and growth rates increased asymptotically, and both responses increased with by incubation time. Our results indicate that WWTP effluent stimulates microbial activities and alters detritivore performance, and stream water dilution may mitigate these effects.

Keywords: WWTP, effluent concentration, community-ecosystem function, microbes, *Echinogammarus berilloni*, subsidy-stress, incubation time

1. Introduction

The world population, the per-capita rate of resource use, the industrial production, the proportion of people living in cities and the number of livestock units in large farms, all are rising steadily (Steffen, Crutzen & McNeill, 2007; Grimm *et al.*, 2008). With them, the amount of pollutants and nutrients entering rivers via point source are also increasing. Nowadays, over 80 % of worldwide wastewater is released directly to the environment without an adequate treatment (UNESCO, 2017). In order to reduce this impact, most countries are investing in wastewater treatment plants (WWTPs). For instance, more than 2500 WWTPs have been put into operation over the last three decades only in Spain (Serrano, 2007). WWTPs have been built to mainly reduce loads of nutrients and organic matter, but they also function to some extent as a filter for other pollutants. However, they are still a major point-source pollution in many river ecosystems (Carey & Migliaccio, 2009; Munz *et al.*, 2017), as their effluents consist of complex mixtures of nutrients, organic matter, metals, and other pollutants, including regulated and emerging pollutants (Petrovic *et al.*, 2002; Gros *et al.*, 2007). Some of these compounds (e.g. pesticides) are toxic and reduce biological activity, whereas others (e.g. nutrients), can subsidize biological activity (Martí *et al.*, 2001; Ribot *et al.*, 2012) up to a concentration threshold, beyond which they also become stressful, a pattern that results in the so-called 'subsidy-stress response' (Odum et al. 1979). Many WWTP effluents are discharged into streams, where allochthonous organic matter such as leaf litter is a key resource for aquatic food webs (Petersen & Cummins, 1974; Tank *et al.*, 2010). Litter decomposition is a complex process in which microbial decomposers and macroinvertebrate detritivores play a leading role (Hieber & Gessner, 2002a). It is highly sensitive to changes in environmental conditions, such as nutrient availability (Niyogi, Simon & Townsend, 2003; Ferreira *et al.*, 2006; Woodward *et al.*, 2012) and the presence of toxic compounds (Lecerf et al., 2006). WWTP-derived nutrients may subsidize microbial activity (e.g. respiration and exo-enzymatic activity) and thus promote litter decomposition (Niyogi *et al.*, 2003; Ferreira *et al.*, 2006). However, excessive concentration of nutrients and other pollutants can be toxic for microbes and macroinvertebrates (Baldy et al., 2007; Camargo and Alonso, 2006; Duarte et al., 2009). Moreover, macroinvertebrate detritivores depend on microbes to condition plant litter and increase its palatability (Bärlocher & Kendrick, 1975; Graça *et al.*, 2001). Thus, direct and indirect effects can alter consumption rates, body condition and death rates of macroinvertebrate consumers (Bundschuh et al., 2013, 2011a). Moreover, if the species sensitive to WWTP effluents are key consumers, such as detritivores that process extraordinary amounts of leaf litter, this pollution might eventually affect rates of ecological processes and the entire food web (Bundschuh et al., 2011b).

Ecotoxicological assays have evolved from those focused on survival facing acute toxicity to assays recording sublethal endpoints, including mobility, carcinogenic effects, hormonal disruption and histopathological, cytological or molecular-level stress biomarkers (Gorokhova *et al.*, 2010; Löf *et al.*, 2016a; Wigh *et al.*, 2017). The results of these assays are essential to rank pollutants according to their environmental risk. Nevertheless, individual stress signals do not always translate into responses at the levels of the community or ecosystem function (Lukančič *et al.*, 2010; Souza *et al.*, 2010;

Trapp *et al.*, 2015). Moreover, results in laboratory experiments cannot be directly transferred to consequences in the field, where biotic interactions and environmental complexity can modulate the responses. Community-Ecosystem Functioning assays (hereafter CEF) are those assays that construct simplified ecosystems in the laboratory where biotic communities (microbes and macroinvertebrates and their interactions) and ecosystem functions are tested for environmental conditions or pollutants. An increasing number of studies show that CEF can respond in contrasting ways to different pollutants and their interactions (Rasmussen *et al.*, 2012b; Zubrod *et al.*, 2015). However, as many factors differ among CEF studies, it is difficult to draw general conclusions or to rank the different toxicants in terms of toxicity as with simpler toxicological studies. One of the varying factors among the studies is the taxa tested. Using standard model taxa as in classic toxicology (e.g. *Gammarus* or *Daphnia*) helps homogenizing studies, but these taxa might not occur in the target ecosystems. Another challenge of CEF studies is the lack of standardized incubation duration. Temporal consistency of the responses has seldom been studied in CEF assays, despite being a stepping stone for these approaches to become more prevalent.

Here, following a CEF approach, we assessed the effect of the concentration of a WWTP effluent on organic matter decomposition and performance of microbes (decomposition, respiration and exo-enzymatic activity) and macroinvertebrate detritivores (food consumption, growth and RNA to body mass ratio). We combined a highly controlled and standardized laboratory experiment with a field bioassay of the effects of WWTP effluents in a stream. The former was used to build an effluent concentration-dependent response model, whereas the later tested the validity of this model in natural ecosystems. We repeated samplings over time in both experiments to assess the consistency of the diagnoses. Our main predictions were: i) microbial and detritivore performance variables will present a subsidy-stress type response along the effluent concentration gradient; ii) differences along the effluent concentration gradient will increase with time, and iii) field measurements will follow patterns observed in the laboratory experiment.

2. Materials and methods

2.1. WWTP characteristics

The selected WWTP (Apraitz) is located in Elgoibar, N Iberian Peninsula (43°13'41" N 2°23'56" W). The facility treats the sewage water of approximately 90,000 population equivalents, mostly urban, but also including industrial sources. An average daily amount of 29.90 m^3 of wastewater is subjected to primary and secondary treatment (http://acciona-agua.com/). An additional tertiary treatment is also carried out, where phosphorus is chemically precipitated via $FeCl_3$ addition. The WWTP is based on sequencing batch reactors in which the sewage water is mixed in large tanks with activated sludge, and alternatively subject to anaerobic and aerobic conditions to reduce organic matter. This process results in pulsed releases of effluent during ca. 20-40 min every 2 h. The effluent is released into the Deba River (av. discharge, 11.4 $m^3 \cdot s^{-1}$) right beside the junction with the Apraitz Stream, a small tributary with average discharge of 0.118 $m^3 \cdot s^{-1}$, and 2.2 m of channel width that flows next to the WWTP and that was used for the field experiment.

2.2. Organic matter and experimental taxa

We used leaf litter of black alder (*Alnus glutinosa* (L.) Gaertner) as the model organic material in the experiment

because it is one of the most common riparian tree species in Europe, it has closely related species that are common throughout the Holarctic region and it has been widely used in decomposition studies in streams (Boyero *et al.*, 2011b; Woodward *et al.*, 2012). Freshly fallen alder leaves were collected in September 2016 and disks (14 mm in diameter) were extracted, air-dried and stored in Petri dishes in a dark dry place until further use. The microbial assemblage used in the experiment was obtained by incubating the organic material on an experimental solution (see below). As the test detritivore we chose an amphipod crustacean; this taxonomic group shows a relative tolerance to pollution and is often key for the detritic pathway of many freshwater ecosystems (Woodward, Papantoniou & Lauridsen, 2008; Besse *et al.*, 2013). The amphipod we selected, *Echinogammarus berilloni* (Catta) is one of the most abundant macroinvertebrate species in the streams of the region (Larrañaga et al., 2009) and has demonstrated to be sensitive to food quality (Larrañaga et al., 2009; Larrañaga et al., 2014). Individuals were kick-sampled (500 µm pore size) in the Apraitz stream, in a reach upstream from the WWTP, where levels of pollution are low but nevertheless some pollutants such as caffeine are detected, what suggests that sampled individuals might be pre-adapted to some degree of pollution. The animals so captured were enclosed individually and carried to the laboratory in stream water. There, we selected individuals with a first thoracic segment length between 0.45 and 0.95 mm (6.40 and 13.62 mm total body length), excluding breeding females, which show a clearly differentiated biochemical composition (Larrañaga et al., 2009).

2.3. Laboratory experiment

For the laboratory experiment, we collected an integrated effluent sample between April 24 and 27, 2017. Each day, 10 L of effluent was collected continuously through the day, filtered (0.1 mm mesh size) into a large container, and then, frozen (-20 ºC) in smaller bottles (see below). Although freezing affects DOC concentration (Fellman, D'Amore & Hood, 2008), because we wanted to change water periodically, we thought it was better to freeze the effluent than to use a different effluent every week or preserving unfrozen the water, as it would change the chemical properties even more (Fedorova *et al.*, 2014; Morosini *et al.*, 2017). The experiment lasted 30 d and water was renewed every week to prevent changing chemical conditions. For the experiment, we prepared a set of microcosms (test tubes 2 cm diameter and 20 cm long, filled with 40 mL of solution, enough to allow free movement of the detritivore) with a range of concentrations (0, 20, 40, 60, 80 and 100 %) by diluting the effluent in filtered (0.1 mm mesh) stream water from the reach the detritivores were collected from.

Alder disks were conditioned in the laboratory to reduce the amount of leachates and to encourage microbial colonization. Conditioning was performed by incubating five alder disks in each microcosm for 15 d at 15 °C, 12:12 light photoperiod and constant aeration. After conditioning (stream water was the only source of microbial propagules), a set of 96 microcosms was kept in incubation for an additional 30 d to measure microbial breakdown. The rest of the microcosms were used for invertebrate performance by adding one individual of *E. berilloni* per microcosm. Microcosms were sampled just after the conditioning for initial values (day 0) and later at days 8, 15 and 30. Replication for each sampling and effluent concentration was of 4 in the microbial incubation (4 replicates × 6 concentrations × 4 sampling times = 96 microcosms in total) and of 10 for the detritivore

incubation (10 replicates × 6 concentrations × 3 sampling times = 180 microcosms in total).

2.4. Microbial performance

The disks incorporated into each microcosm were weighed before conditioning. Initial dry mass of the disks was estimated with a correction factor (air dried-to-leached oven dried) obtained from a set of 4 tubes with 5 disks at each effluent concentration that were subject to the same initial conditioning of 15 d. At each sampling time, 3 out of 5 disks were retrieved from the microcosms, oven dried (70 ºC; 72 h) and ashed (500 ºC; 4 h) to obtain the ash free dry mass (AFDM). The remaining 2 disks were used to measure microbial respiration with a RC650 Respirometer coupled to a SI929-6 Channel oxygen meter (Strathkelvin Instruments, Scotland). The respirometer cells were filled with 3 mL of M9 medium (adapted from Sambrook et al., (2001); Supplementary material C) that was oxygen-saturated. The disks were kept in the respirometer for 40 min with constant agitation, and the last 20 min were used to measure the rate of oxygen depletion in each cell. Additional chambers with oxygen-saturated M9 medium and without disks were used as a control. Oxygen consumption was estimated by the subtraction of the slope in the control cells (mean: -1.33; SE: ± 1.07 $\mu g\ O_2 \cdot h^{-1}$) to the slope of each respirometer cell and corrected for the remaining AFDM of the disks ($\mu g\ O_2 \cdot mg\ AFDM^{-1} \cdot h^{-1}$). The disks used for respirometry were ground and homogenized with an IKA Ultra-Turrax T25 Basic grinder (Saufen, Germany) in 20 mL of M9 medium for exo-enzymatic activity measurements. Microbial exo-enzymes contributing to the degradation of cellulose and hemicellulose (β-glucosidase, BG) and to the acquisition of organic phosphorus (alkaline phosphatase, AP) were assessed following Saiya-Cork et al. (2002). Potential activity of both enzymes was estimated fluorometrically (360 nm excitation, 450 nm emission, 37 °C) using the substrate 4-Methylumbelliferyl β-D-glucopyranoside for BG and 4-Methylumbelliferyl phosphate for AP. Assays were carried out in 96-well microplates and fluorescence was determined with a Tecan GENios microplate reader (Cavro Scientific Instruments, Salzburg, Austria). The required controls and blanks were used to determine autoflorescence and quenching, and the results were expressed as $\mu mol \cdot mg\ AFDM^{-1} \cdot h^{-1}$.

2.5. Detritivore performance

Consumption rates were calculated from the difference between estimated initial dry mass and final weighted dry mass of the 5 alder disks in microcosms with detritivores. *E. berilloni* individuals were photographed at the beginning and at the end of the experiments with a binocular microscope (Leica M165FC, Wetzlar, Germany). From these photographs, the dorsal length of the first thoracic segment (DL) was measured using the "Leica Application suite V4" program (LAS V4.1). Initial and final total body lengths (BL) were calculated using Eq. (1) (Flores *et al.*, 2014a). Instantaneous growth rate (IGR) for each individual was calculated using Eq. (2) (Flores *et al.*, 2014a) where t is time, BL_t is the body length at time t and BL_0 is the initial body length. Finally, by means of the Eq. (3) the body mass (BM) was calculated at the beginning and at the end of the experiment (Flores et al., 2014), and the geometric mean of the body mass was used as the descriptor for each detritivore mass throughout the experiment. The equation fits for the detritivores of the present study it was derived from the same species collected in nearby streams.

Eq. (1) $BL = 14.458 \cdot DL - 0.110$; (mm)

Eq. (2) $IGR = (\ln(BL_t) - \ln(BL_0)) / t$; $(mm \cdot d^{-1})$

Eq. (3) $BM = 0.8213BL - 4.3025$; (mg)

For each individual, the concentration of RNA was quantified fluorometrically on microplates, to assess individual metabolic status. The measurements were performed using RiboGreen to quantify RNA of detritivores after the extraction with N-laurylsarcosine and followed by RNase digestion as described by Gorokhova and Kyle (2002). Fluorescence was measured in a Tecan GENios microplate reader (Cavro Scientific Instruments, Sunnyvale), filters: 485 nm for excitation and 520 nm for emission and black solid flat-bottom microplates. The plate was scanned with 0.2 s $\cdot$ $well^{-1}$ measurement time, with 10 measurements per well at constant temperature (37 ºC).

2.6. Field experiment

To test how well laboratory experiments fit responses in the field, we performed an *in situ* bioassay experiment using a comparable experimental design and measuring the same set of variables. This experiment was conducted from February 28 to May 30, 2017. We experimentally diverted part of the WWTP effluent, thus polluting the lowermost 150 m of the Apraitz Stream before it joins the Deba River. The experiment followed a Before-After Control-Impact Paired (BACIP) design (Downes *et al.*, 2002), which allows detecting the effect of the tested impact while controlling for the effect that temporal and spatial changes could have on the response. Two 100-m long reaches were defined: a Control reach (C) just upstream from the effluent addition point and an Impact reach (I) below that point. At both reaches, the variables were measured Before (B) and After (A) the start of the addition of the effluent to the Impact reach. The WWTP released a mean discharge of 10 L $\cdot$ s^{-1} of effluent into the Impact reach for around 20-40 min every 2 h. The effluent was diluted to 1.6 % on average along the duration of the experiment (stream discharge measured every 5 min by a level-logger ((Solinst Edge 3001; Solinst Canada Ltd., Georgetown)). Fine-mesh bags (20 x 10 cm, 0.5 mm pore size) filled with two alder disks each (24 mm diameter) were conditioned in stream water in the laboratory at 15 ºC (mean temperature in the field: 12.1 ºC) for 15 d. After the conditioning, 20 bags were used to measure microbial performance (5 replicates per sampling day and reach, which were distributed along each reach). Another 60 bags with alder disks and one *E. berilloni* individual in each were incubated to assess detritivore performance (15 replicates per sampling day and reach, distributed along each reach). In each sampling period (Before/After), reach (Control/Impact) and sampling day (Day8/Day15) 20 bags (5 for microbial performance and 15 for detritivore performance) were retrieved (in total 160 bags). The addition started on May 3, with the Before incubation spanning from March 14 to 30 and the After incubation from May 16 to 30.

2.7. Chemical analysis

Effluent conductivity and pH data are continuously measured in the WWTP. During the field experiment, stream water physicochemical characteristics (temperature, dissolved oxygen, pH, oxidation reduction potential (ORP), conductivity and total dissolved solids (TDS)) were measured continuously during 1.5 h (30 m downstream from the effluent input; June 12, 2017) with a multiparametric probe (EXO 2, YSI, USA). Water physicochemical characteristics were also measured in the Control and Impact reaches during the experiment.

Nitrogen and phosphorus concentrations were analyzed in the

integrated sample used in the laboratory experiment and in the samples collected in the field (from the lower end of the Control and Impact reaches and directly from the effluent). Samples were filtered through pre-combusted glass-fiber filters (Whatman International, 0.7 μm) and stored at -20 ºC until analysis. The concentration of soluble reactive phosphorus (SRP) (molybdate method (Murphy & Riley, 1962)) and ammonium (salicylate method (Reardon *et al.*, 1966)) were determined colorimetrically on a UV-1800 UV-Vis Spectrophotometer (Shimadzu, Shimadzu Corporation, Kyoto, Japan). The concentration of nitrate and nitrite were determined with capillary ion electrophoresis (Agilent CE) (Environmental Protection Agency, 2007). Dissolved inorganic nitrogen (DIN) was calculated as the sum of nitrate, nitrite and ammonium.

Additionally, 41 priority and emerging organic compounds (supplementary material A) were analyzed following Mijangos et al. (2018), including herbicides, hormones, life style products (personal care products, stimulants and artificial sweeteners), industrial chemicals (corrosion inhibitors and fluorinated compounds), and pharmaceuticals (antibiotics, tricyclic antidepressants, antihypertensives, anti-inflammatories, β-blocker cardiovascular drugs, lipid-regulating and anticonvulsants), which cover a wide variety of emerging contaminants typically found in WWTP effluents. The selection of the target pollutants was carried out taking into account their presence and relevance in the environment (Busch *et al.*, 2016; Brack *et al.*, 2017; Tousova *et al.*, 2017). Compound families, names, CAS numbers, molecular formulas and other relevant physicochemical properties for all the target compounds are summarized in Supplementary material (A). These analysis were performed on the integrated effluent sample used in the laboratory experiment and in the case of field experiment, spot samples (May15, 2017) were taken from the Control, Impact and WWTP effluent simultaneously every 5 min during 1 h. In both cases (laboratory and field experiment) water samples were kept in the fridge at 4 °C before analysis, which was performed within 24 h according to a previously validated SPE procedure (Mijangos *et al.*, 2018a). Additionally, in the case of the field sample, time weight average concentrations (C_{TWA}) of the stream (Control and Impact) were calculated from May15 to June 12, 2017 by means of passive samplers. At both reaches, a canister containing two polar organic chemical integrative samplers (POCIS) was deployed at ~ 50-100 cm below the surface. POCIS were prepared according to the procedure described by Mijangos et al. (2018b) (sorbent material mixture of 100 mg mixed-mode anion exchange (Strata X-AW) and 100 mg HLB (Plexa) and using a highly porous (30 μm pore size) Nylon membrane sampler). POCIS were transported at -4 °C to the lab and stored at -20 °C before being processed as described in Supplementary material (D).

Emerging organic compound analysis was carried out using a HPLC-QqQ (Agilent 1260 series LC coupled to an Agilent 6430 triple quadrupole) equipped with electro spray ionization (ESI) source (Agilent Technologies) according to a previously optimized method (Mijangos *et al.*, 2018a). The separation of the target analytes was accomplished at a flow of 0.3 $mL \cdot min^{-1}$ using a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 μm) column coupled to a Kinetex F5 pre-column (2.1 mm × 4.6 mm, 2.6 μm). The column temperature and the injection volume were set to 35 °C and 5 μL, respectively. Under optimized conditions, a binary mixture consisting of a mobile phase A of water: MeOH (95:5) and mobile phase B of

MeOH: water (95: 5), both containing 0.1 % of formic acid was used for gradient separation of the target analytes. The gradient profile started with 30 % B which was increased to 50 % in 4 min and maintained for 12 min. Then it was increased to 90 % B where it was maintained constant for 10 min. Initial gradient conditions (30 % B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). ESI was carried out using a nitrogen flow rate of 12 $L \cdot min^{-1}$, a capillary voltage of 3500 V, a nebulizer pressure of 45 psi, and a source temperature of 350 ºC. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection. Quantification was performed in the selected reaction monitoring (SRM) acquisition mode. Fragmentor voltage and collision energy values for each target analyte and the determined apparent recoveries and method limits of quantification (MQLs) are included in Supplementary material (D). Instrumental operations, data acquisition and peak integration were performed with the Mass Hunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

2.8. Data analysis

Consumption of organic matter was calculated by subtracting final AFDM to the initial AFDM values. Initial dry mass was corrected for leaching, and microbial decomposition was removed from the total consumption in test tubes with *E. berilloni* to estimate the consumption by the detritivore. Thus, microbial decomposition was expressed as the depleted AFDM per sampling day. For *E. berilloni* consumption, depleted AFDM was corrected by the dry body mass of the individual per day (mg AFDM · mg BM^{-1} · d^{-1}). The detritivore death rate was calculated per treatment at each sampling day and corrected with the incubation time. Respiration and exo-enzymatic activities were corrected by the remaining AFDM of alder disk at the sampling day. Although RNA:DNA ratio is widely used in the literature (Vrede *et al.*, 2002; Gorokhova, 2003), total nucleic acids and DNA concentrations are determined sequentially from the same sample and RNA concentrations extrapolated subtracting both values. The numerator is thus dependent on the denominator, magnifying error and thus potential variability in the index (Suthers *et al.*, 1996). Therefore, individual RNA concentrations were corrected with the BM of the individuals to obtain the RNA:BM ratio. For the laboratory experiment, we fitted Gaussian models (Madsen & Thyregod, 2010b; Zuur & Ieno, 2010a) as our data accommodated satisfactorily to their requirements. We used these kinds of models to test for the effect of treatment and time on the response variables: microbial decomposition, respiration and exo-enzymatic activity, and detritivore death rate, consumption rate, growth rate and total concentration of nucleic acid. For parameter estimation, restricted maximum log-likelihood (Pinheiro & Bates, 2000) was used, via the lme() function of the package nlme (Pinheiro & Bates, 2016). The fixed structure of the model included treatment (fitted as a continuous explanatory variable, 0-100 % effluent concentration), time (fitted as a discrete explanatory variable, with three levels, Day 8, Day 15 and Day 30) and the interaction between both. The quadratic term of Treatment was also included in the model to test the fit to a subsidy-stress response pattern. A variance structure was also added to the model to deal with observed heterogeneity, allowing different variances per stratum (varIdent(form= ~1|Day)). *In situ* bioassay data were analyzed via linear models, where period (BA) and reach (CI) were treated as fixed factors, and the spatial

variation was fitted as a random factor nested in reaches. The interaction between period and reach was also fitted. The models were fitted using the lm() function of the nlme package (Pinheiro and Bates 2017). Data from days 8 and 15 were analyzed separately, so that the responses for each day were assessed separately. In all cases significance was accepted when $p < 0.05$. All statistical analyses were conducted using R statistical software (version 3.1.2, R Core Team, 2017).

3. Results

3.1. Laboratory experiment

3.1.1. Effluent characteristics

The WWTP effluent had substantially higher conductivity, DIN and SRP concentrations than the stream water (Table 1). Twenty five out of the 41 emerging contaminants analyzed in the integrated effluent sample were above quantification limits, the highest values corresponding to valsartan (26870 $ng \cdot L^{-1}$) and caffeine (14555 $ng \cdot L^{-1}$). Other compounds, such as acesulfame (2610 $ng \cdot L^{-1}$), sucralose (1865 $ng \cdot L^{-1}$), irbesartan (3665 $ng \cdot L^{-1}$), eprosartan (2899 $ng \cdot L^{-1}$) and telmisartan (1637 $ng \cdot L^{-1}$), also exhibited high concentrations (Table 2). In the Control reach, only caffeine was detected by active sampling and 13 analytes with the POCIS (Table 2).

Table 1. Basic water chemical characteristics of the WWTP effluent used during the laboratory and field experiment and the Control reach of Apraitz stream during 2016-2017. Mean values and standard errors are shown. Letters display statistical differences.

Variables	WWTP effluent lab experiment	WWTP effluent field experiment	Stream water
Temperature (ºC)	15_a	20.02 ± 1.13 (n=4)$_b$	14.79 ± 1.07 (n=8)$_a$
Conductivity ($\mu s \cdot cm^{-1}$)	649 ± 18 (n=3)$_a$	765 ± 25 (n=8)$_b$	351 ± 60 (n=8)$_c$
DO (%)	100_a	7.75 ± 3.87 (n=4)$_b$	102.5 ± 1.01(n=8)$_a$
DIN ($mg \cdot L^{-1}$)	8.03 (n=1)$_a$	10.59 ± 2.11 (n=8)$_a$	1.05 ± 0.07 (n=9)$_b$
SRP ($mg \cdot L^{-1}$)	0.77 (n=1)$_a$	1.65 ± 0.25 (n=2)$_a$	0.02 ± 0.01 (n=9)$_b$

3.1.2. Microbial performance

Overall, the effect size of the response was higher for microbes than for the detritivore. The effluent promoted microbial decomposition of organic matter (Treatment, $p = 0.039$, Table 3, Fig. 1), which in the most concentrated treatment increased by 120% for day 30. Neither time nor the time:treatment interaction were significant, but variance decreased from the first sampling to the last sampling. Microbial respiration increased with effluent concentration (Treatment, $p < 0.030$, Table 3, Fig. 1) and time of exposure (Day, $p < 0.001$), with highest values at 100 % effluent concentration and day 30. The interaction between effluent concentration and time of exposure was also significant (Treatment:Day, $p = 0.006$), as the positive relationship was only observed after 15 and 30 d of incubation. Exo-enzymatic activities were also promoted by the effluent concentration (Table 3, Fig. 1). The response of both exo-enzymatic activities to the effluent concentration depended on the exposure time (Day, $p < 0.001$). For AP, the relationship with the effluent concentration only became significant after day 30, for BG was significant after day 15. The quadratic term, which characterizes the hump-shaped curve, was only significant for AP (Treatment^2, $p = 0.026$). The maximum rate of AP activity was predicted to be at 29.4, 42.5, 67.0 and 390.7 % of effluent concentration, at 0, 8, 15 and 30 days of exposure. For both exo-enzymes, the interaction between effluent

concentration and incubation time was significant (Treatment:Day, $p < 0.001$).

3.1.3. Detritivore performance

In total, 51 out of 180 individuals died during the experiment, but the effluent did not affect detritivore death rate (Treatment, $p = 0.464$, Table 3, Fig. 1). The effluent concentration affected the food consumption rate of the detritivore (Treatment, $p = 0.036$, Table 3, Fig. 1). The response followed a hump-shape pattern ($Treatment^2$, $p = 0.037$) across the concentration range, with the highest consumption rates (0.510 mg AFDM · mg BM^{-1} · d^{-1}) at day 8 and 40 % concentration. Food consumption decreased gradually over the experiment (Day, $p < 0.001$). At 100 % effluent concentration it decreased by half (from 0.262 to 0.130 mg AFDM · mg BM^{-1} · d^{-1}) from day 8 to day 30. Growth rate of the detritivore was overall not affected by treatment (Treatment, $p = 0.591$, Table 3, Fig. 1), although the significant quadratic term ($Treatment^2$, $p < 0.041$) showed a significant subsidy response at medium concentrations. Despite reduced food consumption over time, growth rate increased with time (Day, $p < 0.008$). The RNA:BM ratio (as well as RNA concentration, not shown) increased with the effluent concentration (Treatment, $p < 0.001$, Table 3, Fig. 1), but not with time. The overall effect size of the response was lower for the detritivore than for microbes.

3.2. Field experiment

3.2.1. Water quality

In the Before period, temperature, conductivity, dissolved oxygen, DIN and SRP (Table 1), did not differ between Control and Impact reaches. However, within 5 min of effluent discharge, dissolved oxygen, pH and ORP fell, whereas temperature, conductivity and TDS increased (Fig. 2). Dissolved oxygen did not reach hypoxic conditions (i.e. it decreased from approx. 100 % to 80 %) and pH remained neutral (i.e. it decreased from approx. 7.9 to 7.0). Water characteristics returned to initial values after approx. 20 min, when the effluent discharge stopped. This intermittent pattern was repeated every 2 h during the whole study period. The concentration of emerging contaminants measured with the grab sample also varied during one pulse (before effluent input, during input and after input; Supplementary material B). Prior to each effluent discharge, caffeine was the only analyte detected measuring spot samples analysis in both Control (20 ng · L^{-1}) and Impact (39 ng · L^{-1}) reaches. 20 min after the pouring started, 8 more emergent pollutants were detected in the Impact reach (supplementary material B). After pouring, most compounds returned to not detected below quantification levels (Supplementary material B). Moreover, by using POCIS, a technique which allows to detected lower concentrations in comparison with the conventional grab samples approach (Miège *et al.*, 2015), time average concentrations (28 days) in the range of 0.3-128 ng · L^{-1} and 0.6-40 ng · L^{-1} were obtained in the Control and Impact reach, respectively. In the Control reach, the highest concentration corresponded to caffeine (128 ng · L^{-1}), whereas the rest of compounds did not exceed 11 ng · L^{-1}.

In any cases, these values are lower in comparison with previous reported aquatic media affected by wastewater inputs (Guibal *et al.*, 2018; Mijangos *et al.*, 2018b). In the Impact reach, OBT (40 ng · L^{-1}), Irbesartan (37 ng · L^{-1}) and caffeine (34 ng · L^{-1}) were the most concentrated compounds (Table 2).

Figure 1. Laboratory experiment results. Left, microbial performance: ecomposition (mg AFDM · d^{-1}), respiration (µg O_2 · mg $AFDM^{-1}$ · h^{-1}), AP and BG exo-enzymatic activities (µmol · mg $AFDM^{-1}$ · h^{-1}). Right, detritivore performance: food consumption (mg AFDM · mg BM^{-1} · d^{-1}), growth rate (mm · d^{-1}) and RNA:BM ratio (µg RNA · mg BM^{-1}). Lighter to darker symbols represent time from 0 (or 8 if no Day-0 measure available) to 30 d of incubation. Regression lines are drawn with the significant coefficients from model and a single line is represented if incubation time was not significant.

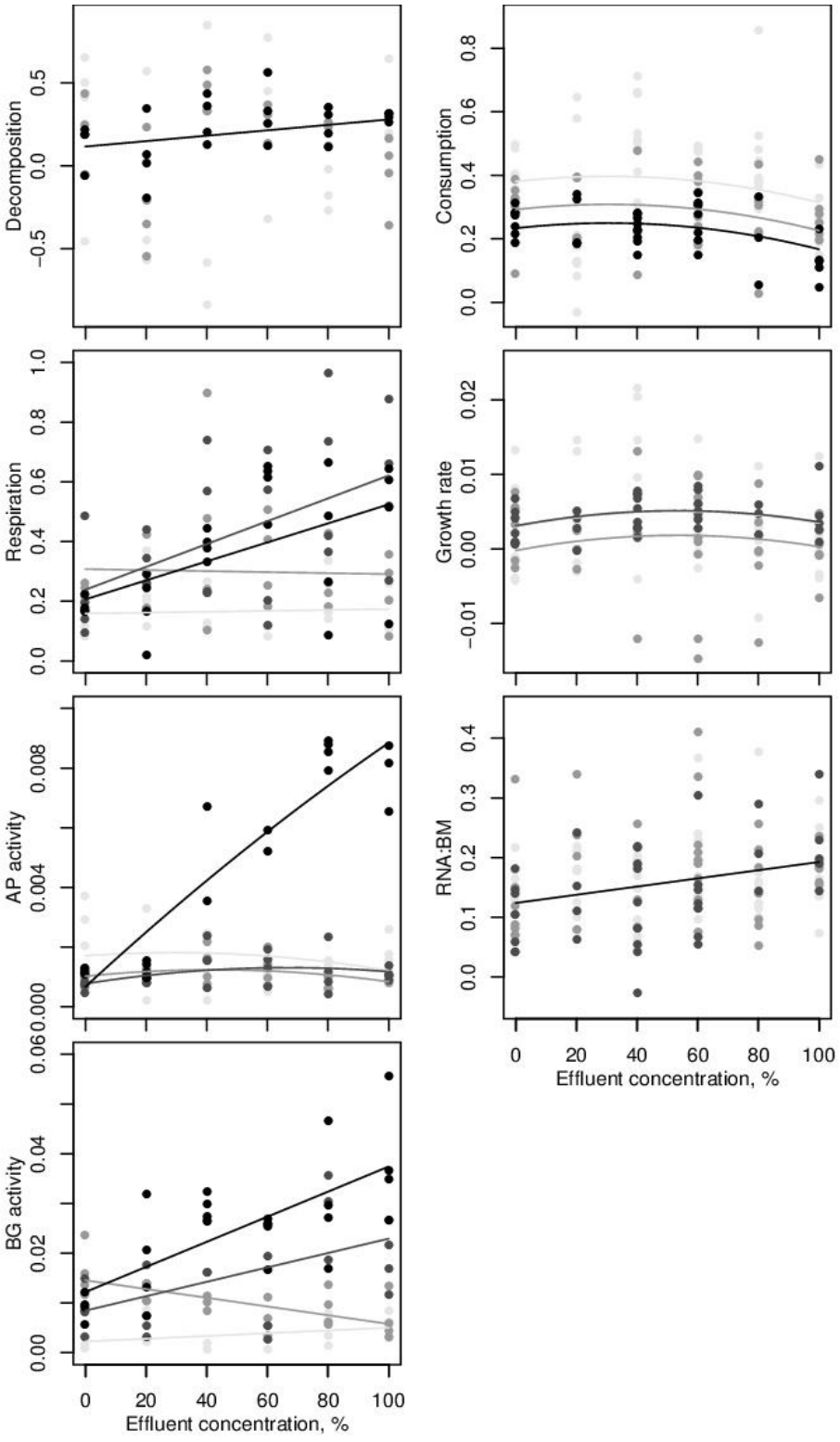

Table 2. Mean concentrations (ng · L^{-1}) of the target analytes determined by means of active sampling for the laboratory experiment (integrated effluent sample) and the field experiment (Control and Impact reach during input and WWTP effluent discharge) as well as the time average concentrations (POCIS, ng · L^{-1}). Mean values and standard errors are shown.

	Laboratory experiment	**Field experiment**				
	Integrated sample	**Spot sample**			**POCIS**	
	WWTP effluent	**Control reach**	**WWTP effluent**	**Impact reach**	**Control reach**	**Impact reach**
Acesulfame	2610 ± 160	n.d.	n.d.	n.d.	n.d.	6 ± 2
Acetaminophen	n.d.	< mql	< mql	< mql	n.d.	19 ± 3.5
Amitriptyline	52 ± 2	n.d.	72 ± 2	26 ± 3	0.9 ± 0.1	6 ± 2
Atrazine	< mql	n.d.	< mql	< mql	1.0 0.1	11 ± 2
Bezafibrate	< mql	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.1
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	14555 ± 373	20 ± 6	76 ± 5	46 ± 6	128 ± 10	34 ± 6
Carbamazepine	104 ± 2	n.d.	122 ± 6	42 ± 3	0.31 ± 0.04	9 ± 2
Ciprofloxacin	100 ± 2	< mql	98 ± 8	44 ± 3	-	-
Clofibric acid	n.d.	n.d.	n.d.	n.d.	-	-
Clomipramine	8.1 ± 0.2	n.d.	12 ± 1	< mql	-	-
Diclofenac	843 ± 3	n.d.	472 ± 61	102 ± 11	-	-
Diuron	325 ± 12	n.d.	267 ± 13	99 ± 12	0.67 ± 0.02	20 ± 5
Eprosartan	2899 ± 63	n.d.	691 ± 65	174 ± 43	-	-
Genistein	n.d.	n.d.	n.d.	n.d.	-	-
Genistin	n.d.	n.d.	n.d.	n.d.	-	-
Glycitin	n.d.	n.d.	n.d.	n.d.	-	-
Imipramine	n.d.	n.d.	n.d.	n.d.	-	-
Irbesartan	3665 ± 33	< mql	3639 ± 71	1161 ± 110	0.5 ± 0.1	37 ± 7
Isoproturon	< mql	n.d.	< mql	< mql	-	-
Ketoprofen	135 ± 3	n.d.	126 ± 10	46 ± 6	0.29 ± 0.03	4 ± 1
Losartan	37.3 ± 0.6	n.d.	14 ± 1	< mql	-	-
Metylparaben	n.d.	n.d.	n.d.	n.d.	-	-
Norfloxacin	34 ± 3	n.d.	34 ± 2	< mql	-	-
Nortriptyline	10.5 ± 0.6	n.d.	13 ± 1	< mql	-	-
Obt	367 ± 15	n.d.	415 ± 16	151 ± 21	10.6 ± 0.8	40 ± 9
Pfbs	260.4 ±0.8	n.d.	147 ± 16	52 ± 11	0.8 ± 0.1	2.5 ± 0.8
Pfoa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pfos	19 ± 3	n.d.	10 ± 1	< mql	0.8 ± 0.2	4 ± 2
Pfosa	n.d.	n.d.	n.d.	n.d.	-	-
Phenytoin	162 ± 24	n.d.	1882 ± 95	549 ± 40	0.3 ± 0.1	12 ± 4
Progesterone	n.d.	n.d.	n.d.	n.d.	0.4 ± 0.1	5 ± 1

Propranolol	34 ± 2	n.d.	35 ± 3	14 ± 1	-	-
Simazine	n.d.	n.d.	n.d.	n.d.	-	-
Sucralose	1855 ± 132	< mql	1977 ± 155	< mql	-	-
Sulfadiazine	5.0 ± 0.3	n.d.	n.d.	n.d.	-	-
Sulfamethoxazole	22 ± 2	n.d.	67 ± 5	37 ± 5	-	-
Telmisartan	1637 ± 111	n.d.	1771 ± 252	341 ± 70	0.38 ± 0.09	4.6 ±0.9
Testosterone	n.d.	n.d.	n.d.	n.d.	-	-
Trimethoprim	56.7 ± 0.3	n.d.	44 ± 3	25 ± 2	-	-
Valsartan	26870 ± 190	n.d.	403 ± 71	< mql	-	-

n.d. non detected; < mql below its limit of quantification; - non included in POCIS calibration

Table 3. Statistical results for the laboratory experiment.

	Microbial performance											
Source of variation	**Decomposition , mg AFDM · d^{-1}**			**Respiration, µg O_2 · mg $AFDM^{-1}$ · h^{-1}**			**AP, µmol· h^{-1} · mg $AFDM^{-}$**			**BG , µmol· h^{-1} · mg $AFDM^{-}$**		
	DF	*F-value*	*p-value*	DF	*F-value*	*p-value*	DF	*F-value*	*p-value*	DF	*F-value*	*p-value*
Treatment	1	4.43	0.039	1	4.86	0.030	1	4.07	0.047	1	0.97	0.328
Day	2	1.17	0.316	3	17.74	<0.001	3	52.07	<0.001	3	61.08	<0.001
Treatment^2	1	1.59	0.211	1	1.42	0.236	1	5.16	0.026	1	2.57	0.113
Treatment:Day	2	0.36	0.699	3	4.48	0.006	3	36.00	<0.001	3	16.99	<0.001

	Detritivore performance											
Source of variation	**Consumption, AFDM · mg BM^{-1} · d^{-1}**			**Instantaneous growth rate, mm · d^{-1}**			**RNA:BM, µg · mg BM^{-1}**			**Death %**		
	DF	*F-value*	*p-value*	DF	*F-value*	*p-value*	DF	*F-value*	*p-value*	DF	*F-value*	*p-value*
Treatment	1	4.49	0.036	1	0.29	0.591	1	12.11	<0.001	1	0.58	0.464
Day	2	31.06	<0.001	2	5.09	0.008	2	0.67	0.512	2	0.98	0.405
Treatment^2	1	4.44	0.037	1	4.29	0.041	1	0.01	0.934	1	0.39	0.545
Treatment:Day	2	3.59	0.060	2	1.55	0.217	2	1.06	0.350	2	0.60	0.577

3.2.2. Microbial performance

Microbial decomposition of alder leaf disks was not affected by the effluent (BA:CI, Day 8; p = 0.146, Day 15; p = 0.572, Table 4, Fig. 3). Neither sampling period nor reach affected microbial decomposition. Microbial respiration only showed differences between the Before and After period at day 8 (BA, p = 0.031, Table 4, Fig. 3). However, the effect of the effluent input on microbial respiration was not significant (BA:CI, Day 8; p = 0.198, Day 15; p = 0.811). Both measured exo-enzymatic activities differed between periods at both sampling days, with higher activities in the Before period (Table 4). However, the effluent did not affect any of the exo-enzymatic activities.

3.2.3. Detritivore performance

Food consumption of detritivores enclosed in bags was not affected by the effluent (BA:CI, p = 0.841, Table 4, Fig. 3), but there were differences between incubation period and reach by day 15 (BA, p = 0.035; CI, p = 0.028). No effect of effluent addition, period or sampling day was detected for growth rate (Table 4). However, the RNA:BM ratio was reduced because of the effluent on both sampling days (BA:CI, Day 8; p = 0.020, Day 15; p = 0.026, Table 4, Fig. 3). This ratio decreased 30.9 % from Control to Impact reach at day 8 and 25.3 % at day 15 (Fig. 3).

4. Discussion

WWTP effluents consist on complex mixtures of compounds, some of which, such as nutrients, are expected subsidize biological activity, whereas others, such as pesticides or many emerging contaminants, are expected to stress biological activity. Therefore, the overall effect of WWTP effluents can depend on their exact composition and final concentration, as well as on the characteristics of receiving water bodies. Many studies have shown that in nutrient-limited streams, nutrient addition promotes microbial decomposition (Suberkropp & Chauvet, 1995; Niyogi *et al.*, 2003; Ferreira *et al.*, 2006). In our case, microbial decomposition was subsidized significantly along the effluent concentration gradient in the laboratory experiment, suggesting a positive nutrient effect. A similar pattern was observed by Biasi et al. (2017), who found that increased nutrient concentrations in laboratory microcosms stimulated microbial activity (e.g. respiration), which was translated into accelerated organic matter decomposition. Similarly, the observed increase in exo-enzymatic activities could be explained by the nutrient enrichment due to the effluent addition (Carreiro *et al.*, 2000). Together with nutrients, the effluent used in the experiment comprised an array of emerging contaminants (i.e. trimethoprim, diclofenac, sulfamethoxazole, sulfadiazine) in the same range as those detected in surveys of WWTP effluents across Europe (Loos *et al.*, 2013; Beckers *et al.*, 2018). In fact, Loos et al. (2013) monitored 90 European WWTPs and found the sartan family to be one of the most relevant emerging contaminant groups with a median concentration of 480 $ng \cdot L^{-1}$, 368 $ng \cdot L^{-1}$ and 227 $ng \cdot L^{-1}$ for irbesartan, telmisartan and eprosartan, respectively. The integrated effluent used in the laboratory experiment exceeded these concentrations up to 4 (telmisartan) or 12 (eprosartan) fold depending on the compound. A variety of the emerging contaminants, and even nutrients above a certain concentration, can have adverse effects on the biota. Leaf decomposing microbial communities are affected by WWTP effluent inputs (e.g. Feckler et al., 2018; Gardeström et al., 2016), although we cannot test this point as we did not analyze microbial communities. Regardless of the changes in community

Figure 2. Physicochemical characteristics in the Impact reach recorded every 5 min during one cycle of the effluent discharge. Temperature (ºC), conductivity (µs · cm^{-1}), total dissolved solid (TDS, mg · L^{-1}), dissolved oxygen saturation (DO sat %), pH and oxidation reduction potential (ORP, mV). The effluent discharge starts at time 0 and lasted 20 min.

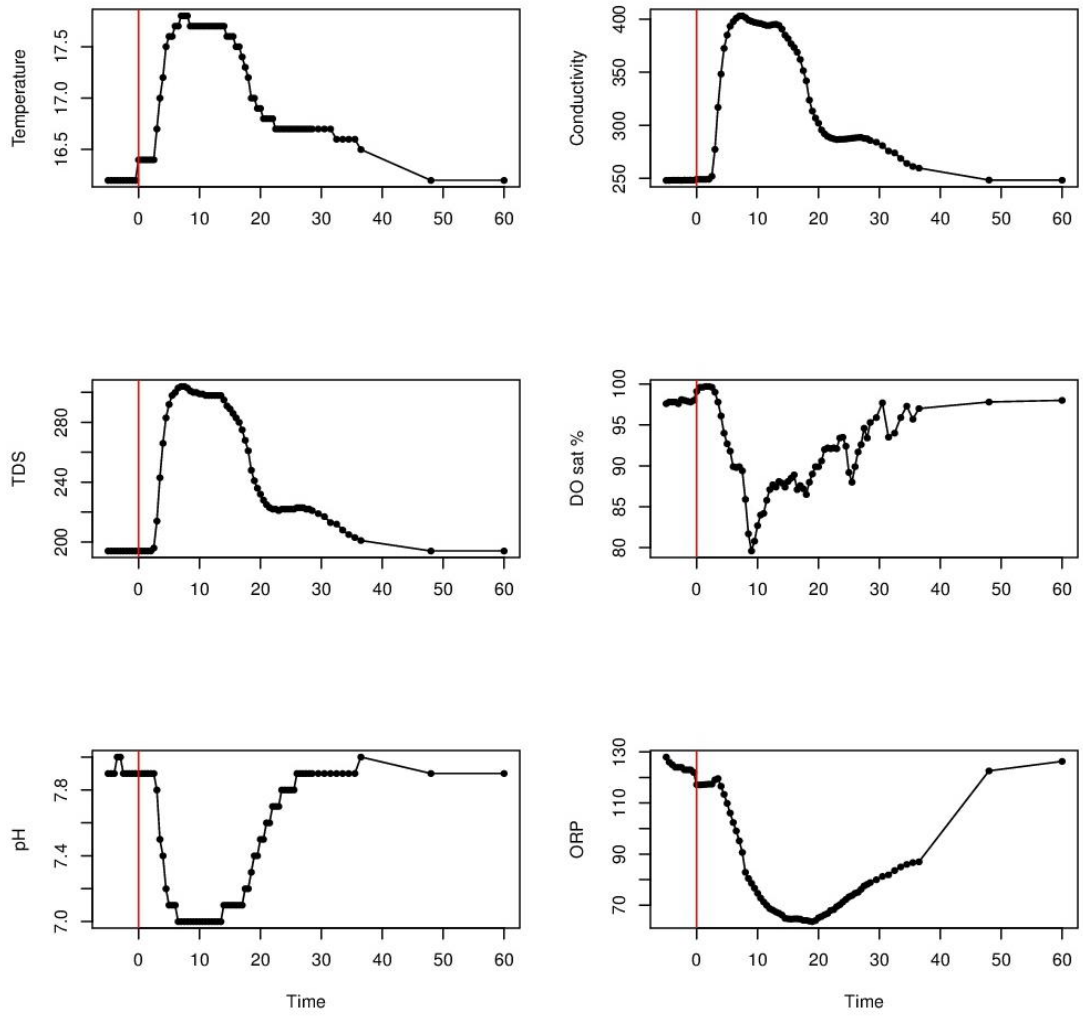

Table 4. Statistical results for the field experiment. BA: Before-After; CI: Control-Impact.

Microbial performance

Source of variation	**Decomposition , mg AFDM · d^{-1}**			**Respiration, µg O_2 · mg $AFDM^{-1}$ · h^{-1}**			**AP, µmol · h^{-1} · AFDM mg^{-1}**			**BG, µmol · h^{-1} · AFDM mg^{-1}**	
	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value
Day 8												
BA	1	2.82	0.115	1	5.71	**0.031**	1	5.45	**0.035**	1	47.93	**<0.001**
CI	1	1.33	0.268	1	3.33	0.089	1	0.19	0.668	1	0.14	0.711
BA:CI	1	2.37	0.146	1	1.82	0.198	1	0.65	0.432	1	0.01	0.942
Day 15												
BA	1	2.27	0.154	1	2.51	0.135	1	35.69	**<0.001**	1	11.41	**0.004**
CI	1	0.41	0.531	1	0.06	0.817	1	0.29	0.597	1	3.37	0.088
BA:CI	1	0.33	0.572	1	0.06	0.811	1	2.74	0.120	1	0.76	0.397

Detritivore performance

Source of variation	**Consumption, AFDM · mg BM^{-1} · d^{-1}**			**Instantaneous growth rate, mm · d^{-1}**			**RNA:BM, µg · mg BM^{-1}**		
	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value
Day 8									
BA	1	0.01	0.911	1	0.38	0.539	1	9.80	**0.003**
CI	1	2.75	0.105	1	0.42	0.520	1	1.64	0.207
BA:CI	1	1.45	0.236	1	1.82	0.184	1	5.90	**0.020**
Day 15									
BA	1	4.87	**0.035**	1	0.98	0.330	1	0.29	0.593
CI	1	5.32	**0.028**	1	0.05	0.819	1	0.01	0.927
BA:CI	1	0.04	0.841	1	0.28	0.598	1	5.51	**0.026**

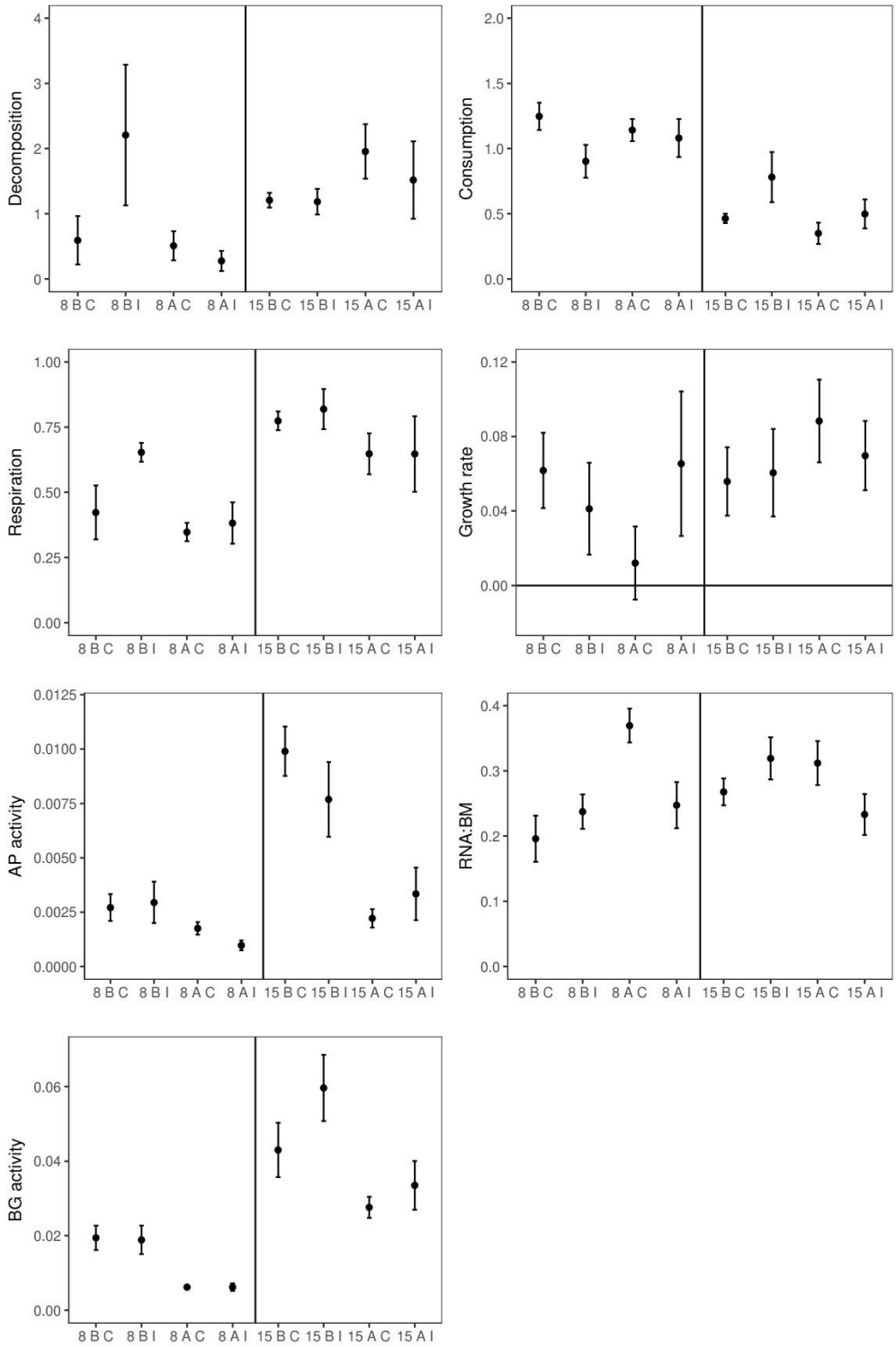

Table 1. Basic water chemical characteristics of the WWTP effluent used during the laboratory and field experiment and the Control reach of Apraitz stream during 2016-2017. Mean values and standard errors are shown. Letters display statistical differences.

composition, in the present study there were no negative effects at the functional level. In this line, Hughes et al. (2016) did not observe any effect on microbial breakdown or detritivore consumption of leaf litter when they were exposed to a mixture of pharmaceuticals. The exact concentration of a cocktail of pollutants at which a certain response variable switches from a subsidy to stress response pattern depends on the species/community (King & Richardson, 2007), and can be especially difficult to predict for complex mixtures of substances as in the case of WWTP effluents (Paine, Tegner & Johnson, 1998; Culp, Podemski & Cash, 2000; Jackson *et al.*, 2016).

Regarding detritivores, in our laboratory experiment, the observed hump-shape response of consumption and growth rate (Fig. 1) did not reach to stress levels as anticipated by our first prediction as none of the effluent concentrations reduced the response below the control treatment. A similar pattern was observed by Woodward et al. (2012), who described an increase in invertebrate-mediated breakdown in European rivers until 18 $\mu g \cdot L^{-1}$ SRP and 1000 $\mu g \cdot L^{-1}$ DIN, followed by a reduction, which did not reach stress values (i.e. values below control). Similar hump-shape responses for detritivore consumption rate were reported by Dunck et al. (2015) and Pereira et al. (2016). Our highest consumption rates were observed at 40 % effluent concentration. Even if the overall effect size (i.e. differences with the control) was small for consumption, the quadratic term of the treatment, which describes the hump-shape response, was statistically significant. *E. berilloni* is a key consumer that has been described in mildly polluted sites (Pérez *et al.*, 2013), and thus, was likely quite resistant to the treatment, especially when, as was our case, the individuals were not collected in a pristine site, which probably pre-adapted them to certain levels of contamination. Moreover, high nutrient concentrations in the field can result in eutrophication and hypoxia, which have direct negative effects on biota. However, the constant aeration in the laboratory experiment avoided hypoxic conditions in the microcosms, thereby increasing the capacity of the animals to resist toxicity.

RNA concentration reflects the rate of protein synthesis, including those produced in detoxification processes (Elser *et al.*, 2000a). In our laboratory experiment, RNA concentrations steadily increased along the effluent gradient (Fig. 1), which might suggest that the effluent was activating the defense systems and repair processes and, with that, protein turnover (Maltby, 1999). For instance, sucralose has been shown to induce neurological and oxidative mechanisms with important consequences on crustacean behavior and physiology (Wiklund *et al.*, 2014). Similarly, propranolol can have negative effects on *Gammarus spp*. physiology (Oskarsson *et al.*, 2012). The increase of RNA concentration observed in our study may have avoided negative implications in the remaining endpoints. Growth rate responded with a significant hump shape (Fig. 1). It has been described that *E. berilloni* individuals can invest more or less in longitudinal growth depending on the environmental conditions (Basset & Glazier, 1995; Glazier, 2000; Larrañaga *et al.*, 2014). We did not study the length-mass relationship of our individuals and growth was derived from body length changes. Thus, we cannot discard that individuals may have switched from longitudinal growth at intermediate concentrations of the effluent to a higher investment in becoming fatter and accumulating more reserves at high effluent concentration. Actually, Larrañaga et al. (2014) noted that mass body condition of *E.*

berilloni tended to maximize at higher nutrient concentrations than longitudinal growth in a laboratory experiment.

In general, the response was clearer in the laboratory experiment than in the field experiment. The only variable that responded significantly to the effluent input in the field experiment, the RNA:BM ratio (Table 4), did it in the opposite direction compared to the laboratory response. This contrasting pattern might be caused by biofilm accrual on top of the bags in the Impact reach (L. Solagaistua, personal observation), which might have reduced the oxygenation of the bags. However, being RNA:BM the only variable that responded negatively, extremely deleterious conditions in these bags are discarded. The generally weaker response in the field experiment was probably caused by the dilution of the effluent in stream water (1.6 % on average along the duration of the field experiment). Even at minimum discharge conditions for the After period in the Impact reach, the effluent concentration never rose beyond 64 %, which would not cause strong responses given the short duration of those peak concentrations. The WWTP effluent was released in pulses, with 20-40 minutes of pouring followed by 100 minutes of non-pouring. In line with this, Nyman et al. (2013) showed that exposure to low and constant insecticide (imidacloprid) concentration reduced feeding and lipid content of *Gammarus pulex*, whereas the same concentration added in repeated pulses did not affect those variables. Similarly, Alexander et al. (2007) observed that the invertebrates *Epeorus longimanus* (insect, Ephemeroptera) and *Lumbriculus variegatus* (annelid, Lumbriculida) could recover from a 1-d exposure to imidacloprid in 4 d. The time required to recover in this study was 4 times longer than the exposure. In our case, the relative recovery time between effluent pulses was 5 times longer than the exposure, and thus, although the effluent could have generated some effects during peak effluent concentrations, the recovery time seems to be enough to avoid changes in the measured variables. Nevertheless, both works cited above assessed the effect of a unique chemical while the effluents used in the present study consist of a mixture of compounds, making direct comparisons complicated.

CEF assays, like the one performed here, have not reached maturity compared to more standardized toxicological assays. Among other methodological differences, the incubation time differs greatly between studies (Englert *et al.*, 2013; Arroita *et al.*, 2016). Our results show incubation time to be a significant factor in the laboratory experiment, as "Day" significantly affected 6 out of 8 measured variables (Table 3). We found that consumption and growth rate of detritivores were significantly affected by incubation time. Both consumption and growth are commonly measured response variables (e.g. Danger et al. 2012; Mas-Martí et al. 2015), whereas incubation time ranges from a week to up to 4 months. There are some generalities that have been pointed out regarding the effect of incubation time on the performance of detritivores. Hessen et al. (2013), for instance, noted that reduced feeding rate along time in microcosm experiments might be caused by decreased energy requirements in laboratory conditions. In our case, we cannot rule out the possibility that reduced consumption was a consequence of decreased food quality over time due to the accumulation of toxicants in the microbial layer. Additionally, in 3 out of 8 variables, incubation time significantly interacted with the treatment (Table 3). In those cases, longer incubations showed clearer differences between treatments, as expected from Haber´s rule. These results mean that the conclusions drawn from the assay depend on the incubation time, which has very relevant implications. Therefore,

time-dependent standardized protocols should be applied to make studies comparable.

When the tested conditions kill the study organisms, this would clearly affect ecosystem function (e.g. consumption). CEF assays enable primarily focusing on sub-lethal effects (e.g. changes in appetite or body condition), that are inherently linked to predicting mid-to-long term effects on processes. From our results, it seems logical then to favor 30 d as preferred incubations for future experiments, as the differences among effluent dilutions were clearer. However, other variables (microbial decomposition and consumer RNA:BM ratio) did not present significant interaction with time, which would make them especially suitable for bio-assessment, as they would yield higher comparability between studies. Besides, longer incubations (months or even years) of macroinvertebrates would include sensitive life stages, and consequently, show impacts that might be more relevant for macroinvertebrate communities in the long-term (Hoguet & Key, 2008). For instance, emerging contaminants released from the WWTP could have affected the reproduction capacity of the detritivore, as pointed out by Wigh et al., (2017), who found that the toxicity of several effluents reduced the fecundity and fertility of *Gammarus fossarum*. Indeed, Englert et al., (2013) found WWTP effluents to affect macroinvertebrate community structure. As amphipod detritivores are very important for organic matter processing and secondary production (Woodward *et al.*, 2008; Piscart *et al.*, 2009), a reduction in fecundity would probably have important consequences for ecosystem function.

CEF assays, as the one described in the present paper, mimic simplified ecosystems as biotic communities (microbes and macroinvertebrates) and interactions between them are included. Measuring the response at different trophic levels increases the ecological relevance of the assay, yielding a better understanding of how ecosystems respond to particular stressors. Moreover, our findings demonstrate that responses are modulated through time and can produce very different acute or chronic effects on ecosystems. However, there is a lack of standardized protocols for CEF assays, which complicates comparisons between studies as well as drawing general conclusions. Therefore, while the effects of new pollutants are measured for different taxa, our efforts should also focus on developing common procedures (e.g. incubation time, measured variables) to have more comprehensive CEF assays in the future.

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Chapter 4

Biomarker response in the amphipod *Monoporeia affinis* to the exposure of a chemical mixture through sediment and food: the difference between acute and chronic exposure

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Abstract

Biota is often exposed to complex mixtures of chemical compounds of anthropogenic origin. Even if the contaminants appear in concentrations below toxicity levels, the mixtures might be detrimental to exposed individuals because of accumulative effects. Moreover, in aquatic environments, sediment acts as a sink as well as a potential long-term source of contaminants rising the basal level of pollutants and exposing the biota to a chronic exposure. On the other hand, biota can also experience acute contaminant exposures due to the pulsed inputs of contaminants. Several biomarkers are used to estimate the biological response to chemical impacts, however, their interpretation is not always straigthforward given the non-linear relationship of some biomarkers with the exposure. In this experiment, we exposed *Monoporeia affinis* individuals to a chemical mixture consisting of hexachlorobenzene, biphenyl, three PCB congeners (52, 101 and 153) and two polycyclic aromatic hydrocarbons (PAHs: pyrene and phenanthrene) in two different scenarios: a) High-Peak treatment, 4 days of high chemical exposure and b) Chronic exposure treatment, 22 days at lower chemical exposure. We compared the responses of the biomarkers to that of Control individuals (22 days of incubation without chemicals). At the end of the experiment oxidative status (ORAC and TBARS), neurotoxicity (AChE) and metabolic status (RNA:DNA, RNA:P and DNA:P) were measured for each individual. We predicted that, i) treatments with the highest concentration-time product would manifest largest effects and ii) correlations between variables responding linearly and non-linearly would assist us in the to diagnosis of the degree of stress that the individuals were subject to. Death rate, TBARS, AChE and DNA:P were not affected by the treatments. As predicted, the High-Peak treatment, which was the one with the highest concentration-time product, was the most detrimental, as suggested by the reduction of ORAC. Exposed individuals reduced their growth rates and seemed to shift to detoxification. Changes in correlation between AChE (linear response) and RNA:P (non-linear response) from Control to exposed treatments demonstrated that correlations are useful tools to determine the stress level in the selected consumers.

Keywords: chemical mixture, sediment contamination, *Monoporeia affinis*, oxidative stress, AChE, biomarker and correlation.

1. Introduction

Increased human activity in the last centuries is responsible for the highly impacted environments in many parts of the biosphere. The perturbations that humankind causes have different origins (e.g., land use change, temperature rise, and chemical pollution) and can appear simultaneously. Among those stressors, effects of pollution on aquatic environments has been extensively studied (for a comprehensive review check Sabater et al., 2018). Most of the studies about risk assessment and management of pollution have focused on single compounds (e.g., Altenburger & Greco, 2009; von der Ohe *et al.*, 2011; Kuzmanović *et al.*, 2015). For example Halm-Lemeille et al., (2014) tested the effects of different PCB congeners individually, which can be very useful to rank the chemicals based on their toxicity. However, biota is most of the time exposed to complex mixtures with varying toxicity and synergetic interactions (Valette-Silver *et al.*, 1999; Sprovieri *et al.*, 2007; Barakat *et al.*, 2013). In the environment each contaminant may be present at concentrations that do not show toxic effects when tested separately in the laboratory; however, toxicity may emerge from chemical mixtures as they rise the baseline toxicity level and extend the exposure in time (Escher et al., 2002; Hermens et al., 1985). For example, Fleeger et al., (2007) observed that while copepod individuals were tolerant to metal and PAH exposures separately, lethality was 1.5 times greater than expected in metal-PAH mixtures.

Many coastal areas in the Baltic Sea suffer from pollution, with high concentration of a variety of contaminants (HELCOM, 2002). The concentration of heavy metals in the Baltic Sea, for instance, is an order of magnitude higher than in the North Atlantic and have not decreased since 1990s (HELCOM, 2003). Moreover, Strandberg et al., (1998) showed that the concentration of contaminants in the Baltic Sea sediments was 5 to 30 times higher than in other seas and oceans. The sediments act as a sink as well as a potential long-term source of different contaminants in aquatic ecosystems. Many persistent organic pollutants (POPs), such as PCBs, are sorbed to organic matter and accumulate in sediments due to their high persistence, hydrophobic nature, and low solubility in water (Nizzetto *et al.*, 2010). As POPs are resistant to degradation, organisms are chronically exposed to these hazardous substances. In addition, wild populations can experience pulse exposures when contaminants are spilled as the increased shipping activities has increased the the occurrence of oil spills (HELCOM, 2003). It has been demonstrated that even small spills (5 m^3 of crude oil) may have direct negative effects on biota (Brussaard *et al.*, 2016). Therefore, the exposure of individuals to pollutants can range greatly in terms of exposure time and concentration; either chronic exposition to low concentrations or acute one to higher loads, with combinations of both being common. To simplify the matter, some researchers have worked with the concept of concentration-time product (CTP), which integrates both variables into one (e.g., Schulz & Liess, 2000; Peterson, Jepson & Jenkins, 2001; Bundschuh *et al.*, 2013).

Beside the toxic effects that different chemicals can have at individual organism level, some pollutants become even more problematic for the food webs, as pollutants may be biomagnified into higher trophic levels across the food chain (Thompson *et al.*, 1989; Bay *et al.*, 1994; Harris, Metcalfe & Huestis, 1994; for a comprehensive review check Gray, 2002). Therefore, in environmental assessment, the early

detection of an impact at a molecular/cellular level (i.e. the use of biomarkers) of low trophic level organisms (e.g. primary consumers) is of paramount importance as early warning detection tools (De La Torre, Ferrari & Salibián, 2005). Biomarkers are variables related to individual status that can be used to estimate the response to environmental stressors (Peakall, 1994). One of the most important features of biomarkers is that a variety of biomarkers can be used in bioassessment, with each one of them delivering information about critical aspects of the physiology of the individual. Biomarkers can be gathered into two groups; some of them detect molecular level damages while others are indicators of the individual´s metabolism, with the latter seeking to repair and detoxify the organism. Biomarkers from the former group are expected to show monotonic responses and thus, to show a linear response along the pollution gradient. On the other hand, metabolic indicator biomarkers will rise or drop (depending on the variable) until a point in which the individual cannot further fight the stress and will start returnign back to basal levels, and thus, they will show hump-shape responses. For example, it has been reported that exposure to polluted sediment and food intensifies the production of reactive oxygen species (ROS) (Livingstone, 2001). Those ROS have direct consequences on individual oxidative status, which defines the balance between pro- and antioxidant processes in the body. The overall antioxidant status can be measured as the Oxygen Radical Absorbance Capacity (hereafter ORAC), which has been found to correlate with various antioxidant enzymes (Gorokhova *et al.*, 2013). Individuals exposed to contaminated sediments increase their capacity to absorb ROS in order to maintain the anti-oxidant/pro-oxidant balance. However, after a threshold, a failure in the anti-oxidant defence system occurs, and individuals are not able to maintain the anti-oxidant/pro-oxidant balance, leading to oxidative stress. This hump-shape response in ORAC capacity was observed by Cortes-Diaz et al., (2017) and is represented in the conceptual framework that we will use for this work (Fig. 1). As the stress builds up there is an effective elimination of ROS at first, but there is an accumulation of ROS when a threshold is surpassed (Timofeyev & Steinberg, 2006). Accumulated ROS products induce damage to macromolecules including lipids, DNA and proteins (Halliwell & Gutteridge, 1999). Hence, as a consequence of exposure to contaminated sediments, and rises on ROS levels, individuals suffer from lipid peroxidation (Barata *et al.*, 2005; Giusto, Salibián & Ferrari, 2014), which is usually measured as thiobarbituric acid-reactive substances (TBARS). Lipid peroxidation is, thus, expected to increase linearly with the stress level as observed by Timofeyev et al., (2006) (Fig. 1). Beside biomarkers related to the oxidative stress (i.e. ORAC and TBARS), assessing the activity of acetylcholinesterase (AChE) is commonly used, as this enzyme regulates the acetylcholine turnover in neuronal synapses. Changes in AChE activity affect several physiological and behavioural processes and can have consequences on feeding, identification and avoidance of predators, and spatial orientation (Pan & Dutta, 1998). Inhibition of AChE activity is a common response to PAH/PCB exposure (Fulton & Key, 2001; Gorokhova *et al.*, 2010, 2013) and it is expected to reduce linearly with increased chemical concentrations (Fig. 1). The cellular levels of nucleic acids (RNA and DNA) and protein, and their ratios (RNA:Protein, DNA:Protein and RNA:DNA) are used as proxies of growth and metabolic status (Dahlhoff, 2004) including the chemical exposure scenarios (Yang, Wu & Kong, 2002; Dahl *et al.*, 2006; Gorokhova *et al.*, 2013). The use of this ratio is based on the

fact that the RNA content of organisms consists primarily on ribosomal RNA. Concentration of ribosomal RNA is directly related to the protein synthesis of a cell (Elser *et al.*, 2000b) and thus, the RNA concentration is directly linked to individual growth (Saiz *et al.*, 1998). In somatic cells, DNA content is quasi-constant and therefore, it is used as a proxy of number of cells (Buckley *et al.*, 1999). Based on this, the use of RNA:DNA ratio is becoming increasingly common to assess growth rates and metabolic status in a variety of aquatic animals (fish: Buckley et al., (1999); copepods: Gorokhova, (2003), Saiz et al., (1998); corals: Meesters et al., (2002) and daphnids: (Vrede *et al.*, 2002)). Additionally, the RNA:Protein ratio is considered a proxy of ribosome number, which represents the absolute upper limit or capacity for protein synthesis (Millward *et al.*, 1973) and the DNA:P can be used as a proxy of cell division status. The three biomarkers related to the content of nucleic acid are directly linked to individual metabolic activity and therefore, it is expected to observe a hump-shape response along the stress gradient.

The use of biomarkers in field and laboratory studies is complicated because there is controversy over how biomarker responses should be interpreted (Sarkar *et al.*, 2006). On the one hand, if the tested impact is not strong enough, we might not be able to detect responses due to high natural variability found in wild individuals such as gender, age, size, behaviour or physiology (Bolnick *et al.*, 2011). Those individual biotic factors considerably affect the biomarker response (Sukhotin, Abele & Pörtner, 2002). On the other hand, non-linear responses of biomarkers (e.g., hump-shape response shown in Fig. 1) could lead to the same responses at low and high chemical concentration exposures, which might lead to incorrect conclusions if the response is not well characterized through different concentrations. For example, following the conceptual framework for the present work (Fig. 1), the measurement of ORAC values at "a" and "b" areas results on the same response and therefore, without more information, we could conclude that tested individuals are not stressed.

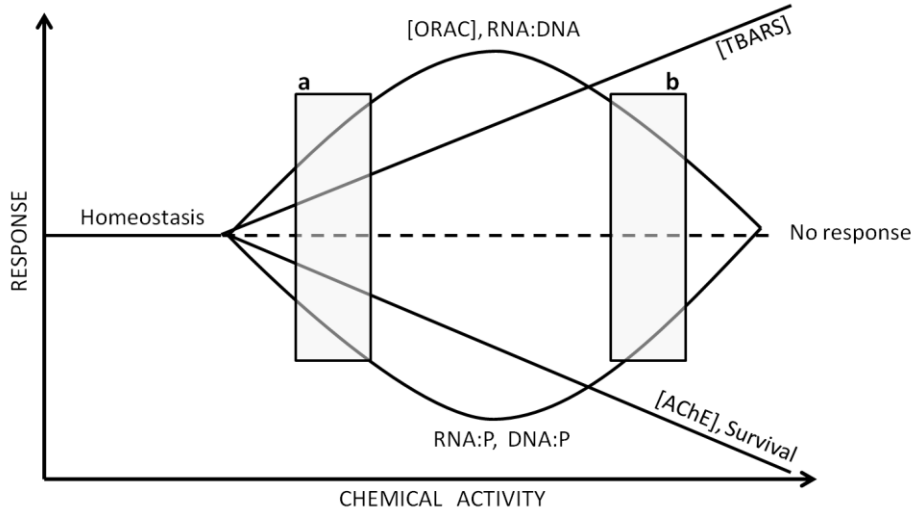

Fig. 1. Conceptual framework for the response of the measured biomarkers. Dashed line indicates no response. The "a" and "b" areas show similar response values for ORAC but the correlation between ORAC and TBARS is reversed. Note that for *a* and *b* we have not described a point because we expect a degree of inter-individual variability in the response.

However, studying a series of biomarkers at the same time can assist us on identifying the position we are within the pollution gradient, as non-linear response biomarkers reverse their correlation with linear response biomarkers along the gradient stress (Fig. 1). For instance, at "a" area, ORAC presents a positive correlation with TBARS while this correlation is inversed to negative at "b" area. In those cases in which we are unable to detect a response using single biomarkers, the measurement of a battery of biomarkers and their correlations will increase our ability to successfully detect and measure environmental responses to chemical impacts.

In this study, we tested the effects of a mixture of chemical contaminants (hexachlorobenzene, biphenyl, PCB52, PCB101, PCB153, pyrene and phenanthrene) at two different exposure scenarios in the amphipod *Monoporeia affinis* using a battery of biomarkers. We selected *M. affinis* as a model species because this sediment-living amphipod is one of the key species of the benthic communities in the Baltic Sea. In the experiment, *M. affinis* was reared in three different scenarios: (1) High-Peak treatment, (2) Chronic exposure treatment and (3) Control treatment. The different treatments aimed to mimic ecologically relevant exposure scenarios, i.e., sudden exposure to a concentrated contaminant spill and a continuous contaminant exposure to basal concentrations. We predicted that i) treatments with the highest concentration-time product would manifest larger effects and ii) correlations between variables responding linearly and non-linearly would enable us to diagnose better the degree of stress that the individuals were subject to.

2. Materials and methods

2.1. Sampling site and collection

The deposit-feeding amphipod *M. affinis*, a sentinel species used as an indicator of the biological effect of contaminated sediments in the Swedish National Monitoring program, was selected as the test organism. Amphipods and sediment were collected outside the Askö field station (58º46´ N, 17º46´ E, station 6022) during November 2016. Young of the year individuals (not sexually mature) were selected because during the sampling campaign mature females were gravid and males die after spawning. The sampling site had a water depth of 46 m and is included as a reference site in the Swedish national environmental monitoring program. A benthic sled was used for amphipod and sediment collection; the sled was set to collect the top 3 centimeter of the sediment. After collection, amphipods were transported in boxes with sediment and sea water to the laboratory. Prior to experiment, the sediment was sieved (mesh size of 0.5 mm) with some water to remove all macrofauna. *M. affinis* individuals were kept in transport boxes with constant air bubbling at 5 ºC and in darkness.

2.2. Experimental setup

The experiment was conducted in 1 L glass jars. To each jar, 75 g of wet sediment were added (approximately 1 cm sediment layer). Thereafter, 600 mL brackish water (practical salinity unit 6.1) was added and the system was left to settle prior to starting the experiment. During the experiment, amphipods were fed with a food slurry consisting of a mixture of fish flakes (1.94 C $g \cdot m^{-2}$) and the algae *Pseudokrichinella suspicata* (0.31 C $g \cdot m^{-2}$). The algae *P. subcapitata* were grown on MBL medium at constant illumination (70 $\mu E \cdot cm^{-2}\ s^{-1}$) and temperature (24 ± 2 ºC). The food slurry was added on top of the sediment 1

day before the start of the incubation. Just before beginning the experiment, 20 individuals were picked up and snap frozen to be used as descriptors of the initial individual condition. The experiment was initiated when *M. affinis* individuals were picked up and transferred to the experimental jars (density: 2760 individuals · m^2). The experiment was carried out at darkness, with constant temperature (5 ºC) and constant aeration in order to simulate field conditions. Individuals were subject to a mixture of contaminants in different scenarios: (1) in the "High-Peak treatment" individuals were incubated at control condition during 18 days followed by 4 days of exposure to high chemical concentration, (2) "Chronic exposure treatment" individuals were exposed to low chemical concentration for 22 days, and (3) "Control treatment" in which individuals were incubated for 22 days without any chemicals (check the nominal concentrations below). Each treatment was done in 5 replicate jars and within each jar 16 individuals were incubated.

2.3. Chemicals

The chemical mixture consisted on hexachlorobenzene (HCB), biphenyl, three different PCB congeners (52, 101 and 153) and two polycyclic aromatic hydrocarbons (PAHs), pyrene and phenanthrene with nominal concentrations described in Table 1. Those chemical compounds have been widely described in the sediments of the Baltic Sea (Baumard *et al.*, 1999; Sobek *et al.*, 2015). We aimed to achieve two exposure scenarios; the chronic exposure that individuals could suffer in the Baltic Sea and the High Peak treatments which simulate a sudden spill of contaminants. Sediment was spiked, i.e. exposed to chemicals, by vertically rolling the chemicals dissolved in a solvent in brown 1 L glass jars (Ditsworth, Schults & Jones, 1990). The solvent was then allowed to evaporate and the chemicals adsorbed to the inner walls of the glass jar. Prior to solvent evaporation, dimethyl sulfoxide (DMSO) was added (0.45 mL) as a keeper to each jar to retain volatile compounds. The walls of the glass jars were first loaded with 3 mg · mL^{-1} stock solution of HCB dissolved in acetone:hexane (1:3 v/v). After solvent evaporation the second stock solution (3 mg · mL^{-1} biphenyl, 2 mg · mL^{-1} PCBs and 2 mg · mL^{-1} PAHs dissolved in acetone) was added to the jar walls and solvent was evaporated again. Control jars were handled similarly by adding corresponding amounts of DMSO and solvents. Thereafter wet sediment collected in the field was added to the jars (450 g · jar^{-1}), they were covered with aluminium foil and sealed with the lid. The sediments were rolled in the jars for 12 days at 20 ºC. Due to the limited space in the roller mixer, the jars were rolled in batches of two, with approximately 12-hour intervals, and kept in the vertical shaking table meantime. All jars were opened daily and aerated with a glass pipet for a minimum of 30 min.

Table 1. Nominal concentrations spiked to the sediment (dry weight).

	Treatment		
mg/Kg	**Control**	**Chronic exposure**	**High-Peak**
HCB	0	10.93	679.99
Biphenyl	0	4.00	670.38
PCB52	0	2.67	446.69
PCB101	0	2.67	447.73
PCB153	0	2.67	446.5
Pyrene	0	2.67	447.31
Phenanthrene	0	2.66	446.57

The fish flakes were spiked with the chemical mixture (same organic carbon-based concentration as in the sediment, see supplementary material A) in order to avoid dilution of the spiked mixtures when fish fakes were added.

Sediment samples for chemical analysis were collected prior the experiment (before distributing the sediment in the microcosms; day 1) and at the end of the experiment (day 22). Samples within the treatment were pooled at the end of the experiment prior to the analysis. In the sediment samples, we measured the total concentrations of each spiked chemical. We also estimated the amount of chemical that was bioavailable for invertebrates (Jahnke et al., 2014; Mäenpää et al., 2015), for that, we quantified the freely dissolved concentrations (C_{free}) in the sediment porewater (Mayer *et al.*, 2014). See supplementary material A for further information of how concentrations were determined. As an integrative measure for the exposure to chemicals the chemical activity (Reichenberg & Mayer, 2006) of the mixture ($CA_{mixture}$) was calculated according to Eq. (1):

$$\text{Eq. (1)}\ CA_{mixture} = \sum \left(\frac{C_{free}}{C_w^{sat}}\right)_1 + \left(\frac{C_{free}}{C_w^{sat}}\right)_2 + \cdots + \left(\frac{C_{free}}{C_w^{sat}}\right)_n$$

where C_{free} is the freely dissolved porewater concentration and C_w^{sat} is the subcooled liquid solubility (i.e solubility in water, Schwarzenbach et al., 2003) of each respective chemical (1→n) of the mixture. Chemical activity represents the potential of partitioning of the compound into the organisms (Mayer & Holmstrup, 2008).

In order to compare the effect of exposures differing in concentration and time, the concentration-time product (CTP = concentration*time) can be used to integrate both values. The concentration-time product (CTP) of the exposure was calculated for the High-Peak and Chronic exposure treatments. CTP was not calculated for the Control treatment because individuals were not subject to toxic exposure. CTP was calculated twice 1) based on sediment measured concentrations (mg · Kg^{-1}), and 2) based on freely dissolved concentrations (mg · L^{-1}). For each chemical compound a CTP value was calculated by multiplying concentration and exposure time (i.e., 4 days in the High-Peak treatment and 22 days in the Chronic exposure treatment). The difference between both treatments CTP was then calculated for each compound (CTP ratio) and a mean CTP difference calculated for sediment based concentrations and freely dissolved concentrations. Tables with the results are showed in supplementary material B.

2.4. Biomarker analyses

At the end of the experiment, each experimental unit was sieved, and the animal survival was noted. Live amphipods were transferred to Eppendorf tubes individually, snap frozen and stored at - 80 ºC until analysis. Each amphipod was homogenized in 450 µl of cold (4 ºC) buffer (pH 7.2; 50 mmol · L^{-1} tris(hydroxymethyl)aminomethane (Tris) buffer, 0.15 mol · L^{-1} NaCl, 0.3 mol · L^{-1} sucrose, 1 mmol · L^{-1} ethylenediaminetetraacetic acid (EDTA), 0.02 mol · L^{-1} NaH_2PO_4, and 0.1 % Triton X-100; (Schreck *et al.*, 2009)) with glass-beads for 3 × 20 s using FastPrep homogenizer. The homogenate was centrifuged at 10,000 rpm for 5 min at 4 ºC, aliquoted, and frozen at - 80 ºC.

All biomarker analyses described below were conducted using microplate reader FLUOstar Optima (BMG Lab Technologies, Germany) with absorbance (protein and AChE) and fluorescence

(ORAC, TBARS and nucleic acids) configurations. All samples, standards and blanks were analysed in duplicates and the average value was used in the statistical analyses.

2.4.1. Protein concentration

Protein concentration ($\mu g \cdot mL^{-1}$) in the homogenate was measured using the bicinchoninic acid assay (BCA, Pierce Ltd.) with bovine serum albumin as standard. At each well the absorbance was measured at 540 nm, with integration time of 1 s and 20 measurements per well.

2.4.2. Oxygen Radical Absorbance Capacity

The total antioxidant capacity was determined using Zen-Bio ORAC Antioxidant Assay Kit, measuring the fluorescein fluorescence decay over time due to peroxyl-radical formation by the breakdown of AAPH (2,2´-azobis-2-methyl-propanimidamide, dihydrochloride). Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] served as a positive control inhibiting fluorescein decay in a dose dependent manner. ORAC values were were normalized for protein content in the homogenate and expressed as mg Trolox $\cdot$ mg protein^{-1}.

2.4.3. Lipid peroxidation status

Lipid peroxidation was measured by a modified TBARS method were thiobarbituric acid reactive substances (TBARS) formed during lipid decomposition react with thiobarbituric acid (TBA) forming a pink colored product from which the fluorescence intensity was measured ($\lambda_{ex/em}$ = 544nm / 590nm). TBARS were normalized for protein content in the homogenate and expressed as pmol $\cdot$ mg protein^{-1}.

2.4.4. Acetylcholinesterase

AChE activity was measured using acetylthiocholine iodide (AcSCh 75 mM) as a substrate and dithiobisnitrobenzoate (DTNB 10 mM) as a reagent at 20 ºC. Changes in absorbance (405 nm) were measured every 2 min for 10 cycles. The activity values were normalized for protein content in the homogenate and expressed as nmol AcSCh $\cdot$ min^{-1} mg protein^{-1}.

2.4.5. Nucleic acid content

The nucleic acid extraction was performed with 1 % N-laurylsarcosine in TE buffer. Individual RNA and DNA concentrations were quantified using microplate fluorometric high-range RiboGreen (MolecularProbes, Inc.,Eugene, OR) assay (Gorokhova & Kyle, 2002). After the first fluorescence reading at $\lambda_{ex/em}$ = 485 nm / 520 nm providing measure of the total fluorescence (RNA + DNA), 10 µL of RNase were added followed by a second reading to estimate DNA. Different ratios were calculated with the nucleic acids and protein concentrations: RNA:DNA, RNA:Protein and DNA:Protein as proxies of growth and metabolic status, protein synthesis efficiency and cell division status, respectively.

2.5. Data analysis

Death rate was calculated for each incubation unit using Eq. (2),

Eq. (2) $Death\ rate = (\ln(T_0) - \ln(T_x)) / Day.$

where T_0 is the number of individuals at the beginning of the experiment, T_x the number of individuals at the end of the experiment and Day corresponds to the exposure duration.

For the statistical analysis of death rates, incubation jar was the basic data unit and thus, we had 5 death rate values for each treatment. For the rest of the variables

(ORAC, TBARS, AChE, RNA:DNA, RNA:P and DNA:P) the individual was the basic data unit for the statistical analysis, as all the biomarkers were measured for each individual, but correlation of individuals being grown in the same jar was taken into account in the analyses (see below).

Chemical mixture exposure time in the High-Peak treatment lasted 4 days after the initial incubation of 18 days at Control conditions. Thus, chemical exposure time was different at High-Peak and Chronic exposure treatments (4d vs. 22d). Therefore, we wanted first to test the effect of incubation time on Control individuals to assess its suitability for comparisons between treatments. To test that, we performed linear mixed effect models from package lmer() for each response variable (biomarkers), where sample type (T_0 individuals vs. individuals collected after 22 of incubation in Control treatment) was fitted as the explanatory variable and the incubation unit (jar) as a random factor. The results of the statistics are shown in supplementary material "C". Incubation time did not affect the response of any of the measured variables at Control treatment.

A principal component analysis (PCA) was performed using the entire dataset for all the measured biomarkers (ORAC, TBARS, AChE, RNA:DNA, RNA:P and DNA:P) to evaluate the intercorrelation of the measured variables. The PCA was followed by a redundancy analysis via the rda() function of the package vegan (Jari Oksanen *et al.*, 2018) to test for the effect of the Treatment (Control, Chronic exposure or High peak) on the multivariate response of the biomarkers. The redundancy analysis was based on Euclidean distances. All variables were log transformed prior the PCA and rda analyses to work with simetric distributions and avoid the excessive influence of extremely large values on the analyses.

To test the effect of treatment (Control, Chronic exposure or High-Peak) on each response variable measured in *M. affinis* (ORAC, TBARS, AChE, RNA:DNA, RNA:P and DNA:P) we fitted Gaussian models (Madsen & Thyregod, 2010a; Zuur & Ieno, 2010b). The fixed structure of the models included treatment (fitted as a discrete explanatory variable, with three levels, Control, Chronic exposure and High-Peak) and incubation unit (jar) was included as a random factor. For parameter estimation, linear mixed-effect models were used, via the lmer() function of the package nlme (Pinheiro *et al.*, 2018). Analysis of Variance (ANOVA) was performed on these models to test for the significance of the Treatment. The effect of the Treatment on death rate was tested with linear models lm() folowed by ANOVA, as we had a single death rate value per incubation unit. Data from all tested responses were tested for normal distribution and homogeneity of variances by means of residual analyses. AChE values did not show the required normal distribution and in order to normalize the data a log-transformation was required. Turkey's HSD tests were carried out for post-hoc pairwise comparisons between Control, Chronic exposure and High-Peak treatments.

Pearson's product moment correlation coefficients were calculated to check correlations of death rates with biomarkers. Correlations were performed with mean values of each incubation unit. In order to test the effect of Treatment on the correlation between the biomarkers, linear mixed effect models (lmer) were fitted. P values were obtained by means of likelihood ratio tests comparing models with a different amount of sources of variation (Pinheiro & Bates, 2000). The structure of the main model included the covariable (biomarker), Treatment and the interaction between both sources of

variation as the fixed factors, as well as the incubation unit (jar) as the random factor.

In all cases significance was accepted when $p < 0.05$. All statistical analyses were conducted using R statistical software (version 3.1.2, R Core Team, 2018).

3. Results

3.1. Chemicals

Overall, the exposure concentration of the chemicals mixture was constant throughout the experiment (Table 2), indicating that an equilibrium between chemicals and sediment was reached during the spiking process. However, in the Chronic exposure treatment, pyrene concentration decreased 64 % from the start to the end of the experiment and phenanthrene 33 % (Table 2). Low levels of HCB, PCBs and pyrene were also detected in the Control treatment sediment at the end of the experiment (Table 2). For further information check supplementary material A.

Freely dissolved concentrations ranged from 0.00002 mg · L^{-1} PCB 153 and 0.00281 mg · L^{-1} pyrene in Chronic exposure to 0.00042 mg · L^{-1} PCB 153 and 0.14482 mg · L^{-1} pyrene in High-Peak treatment (Table 3). For PCB 52 the observed C_{free} was higher than expected in comparison to the other compounds determined (Table 3). C_{fee} for Phenanthrene and Biphenyl were not calculated as polymer-water partition coefficients were not available for these compounds.

The CTP in the sediment was 10 times higher in the High-Peak treatment compared to the Chronic exposure treatment on average (it varied from 8.15 to 12.92 depending on the compound; supplementary material B, Table SP1). The mean CTP of freely dissolved chemicals (i.e., bioavailable chemicals) was 6.4 times higher in the High-Peak treatment than in the Low-Long treatment. The difference in CTP of freely dissolved chemicals between both treatments ranged from 1.15 for HCB to 10.34 for PCB 52 (supplementary material B, Table SP2).

2. Ecotoxicological and biomarker responses

The death rate per incubation jar was not significantly affected by the experimental treatments ($p = 0.581$, Table 4). One replicate from the High-Peak treatment showed a substantially higher death rate; however, this outlier did not affect the overall pattern.

Regarding the multivariate analysis, the first two principal components (PC) explained 56.91 % of the total variability, with the first principal component (PC1) representing 33.15 % of the total variability and the second (PC2) 23.76 %. The correlation pattern revealed that TBARS and AChE were positively related while ORAC and DNA:P responded in opposite directions. The redundancy analysis did not support the Treatment as an explanatory variable for the multivariate response of the biomarkers observed (rda, *F*-value = 1.63; *p*-value = 0.123).

Table 2. Mean values (min-max) of the determined total sediment concentrations (mg · kg^{-1} sediment dw, n = 2) prior experiment (Start) and at the end of the experiment (End).

Treatment	**Control**		**Chronic exposure**		**High-Peak**	
mg · kg^{-1}	**Start**	**End**	**Start**	**End**	**Start**	**End**
HCB	nd	0.05 (0.01-0.09)	3.95 (3.25-4.65)	3.10 (1.34-4.87)	136 (131-142)	169 (155-184)
Biphenyl	nd	nd	nd	nd	nd	nd
PCB52	nd	0.01 (0.01-0.01)	2.85 (2.63-3.08)	3.55 (2.46-4.63)	256 (249-263)	252 (246-258)
PCB101	nd	0.01 (0.01-0.01)	3.54 (3.29-4.80)	3.79 (2.70-4.89)	303 (300-307)	240 (206-275)
PCB153	nd	0.05 (0.01-0.09)	2.78 (2.57-3.00)	3.00 (2.19-3.81)	256 (242-270)	181 (155-208)
Pyrene	nd	0.03 (0.02-0.03)	2.52 (2.02-3.03)	1.63 (1.47-2.14)	101 (98-104)	108 (97-118)
Phenanthrene	nd	nd	1.81 (1.47-2.14)	0.60 (0.41-0.80)	27 (26-28)	27 (19-34)

Table 3. Freely dissolved chemical concentrations (C_{free}, mg · L^{-1}; mean v± standard deviation) of each chemical and in each treatment as well as chemical activity of the mixtures ($CA_{mixture}$). Polymer-water partition coefficients were not available for Phenanthrene and Biphenyl and thus, C_{free} could not be calculated.

Chemical		**Treatment Cfree** (mg · L^{-1})		
	n	**Control**	**Chronic exposure**	**High-Peak**
HCB[b]	6	nd	0.00126 ± 0.00049	0.00797 ± 0.00276
Biphenyl				
PCB52[a]	4	7.98E-06 ± 3.00E-06	0.00215 ± 0.00073	0.12263 ± 0.03242
PCB101[b]	4	nd	0.00006 ± 0.00002	0.00243 ± 0.00057
PCB153[b]	4	1.19E-08 ± 5.31E-09	0.00002 ± 0.00001	0.00042 ± 0.00010
Pyrene[a]	6	8.34E-06 ± 2.61E-06	0.00281 ± 0.00089	0.14482 ± 0.05373
Phenanthrene				
$CA_{mixture}$		0.0001	0.21	3.6

a) Polymer-water partition coefficients from Mustajärvi et al. 2017, b) Calculated polymer-water partition coefficients from Gilbert et al. 2015.

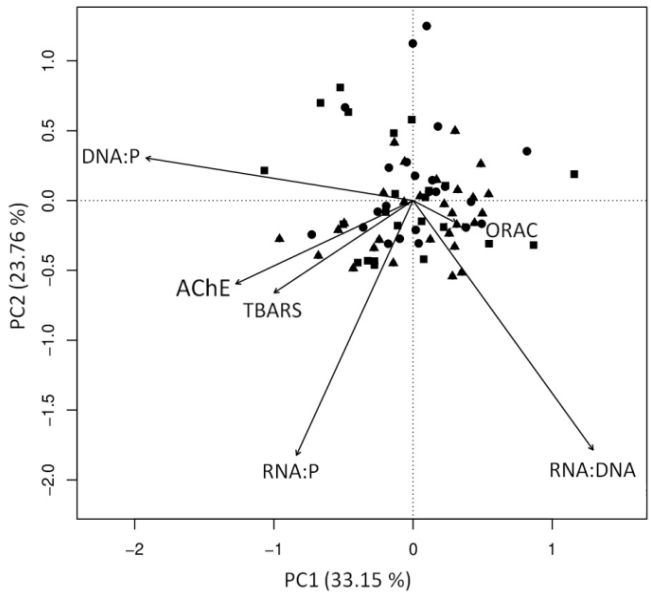

Fig. 2. PCA biplot. Projection of the variables on the factor plane. Symbols: "■" Control, "●" Chronic exposure treatment and "▲" High-Peak treatment.

Table 4. ANOVA results for the effect of the treatment on the measured response variables of *M. affinis*. Results from linear mixed effect models and Turkey's HSD post-hoc pairwise comparisons are represented. Significant responses ($p < 0.05$) are in bold face.

Variable	ss	df	ms	*F*-value	*p*-value	Tukey´s HSD		
						High-Control	Chronic-Control	Chronic-High
Death rate	0.001	2	0.001	0.58	0.581	0.692	0.986	0.595
ORAC	0.013	2	0.007	4.83	**0.024**	**0.006**	0.552	0.119
TBARS	0.083	2	0.041	0.61	0.562	0.530	0.706	0.959
AChE	0.006	2	0.003	0.10	0.905	0.963	0.982	0.897
RNA:DNA	0.155	2	0.078	3.59	**0.032**	0.093	0.898	**0.038**
RNA:P	0.206	2	0.103	4.15	**0.019**	0.999	**0.033**	**0.027**
DNA:P	0.059	2	0.029	1.46	0.239	0.290	0.313	0.999

Focusing on each variable separately, the ORAC of the individuals was significantly affected by the treatment (p = 0.024, Table 4, Fig. 3). The post-hoc analysis revealed that the difference between High-Peak and Chronic exposure treatment was not significant (p = 0.119; Table 4, Fig. 3), whereas a significant decrease of 30 % was observed from the Control to the High-Peak treatment (p = 0.006; Table 4, Fig. 3). The TBARS and AChE were not affected by the chemical exposure treatments (Table 4). The RNA:DNA ratio of the individuals was significantly affected by the treatments (p = 0.032, Table 4, Fig. 3). More specifically, none of the chemicals exposure treatments differed from the Control treatment but the RNA:DNA ratio in the High-Peak treatment (mean RNA:DNA = 0.546) was

significantly higher (p = 0.038, Table 3) than in the Chronic exposure treatment (mean RNA:DNA = 0.457). The RNA:P ratio was also significantly affected by the experimental treatments (p = 0.019, Table 4, Fig. 3). Individuals from the Chronic exposure treatment presented significantly lower RNA:P ratios than individuals from the Control treatment (p = 0.033, Table 4, Fig. 3), however, the response of individuals exposed to the High-Peak treatment did not significantly differ from Control individuals (p = 0.999, Table 4, Fig. 3). The difference in RNA:P between Chronic exposure and High-Peak treatments was significant (p = 0.027, Table 4, Fig. 3), the individuals from Chronic exposure treatment presented lower RNA:P ratio. The DNA:P ratio was not affected by any of the treatments (p = 0.239, Table 4).

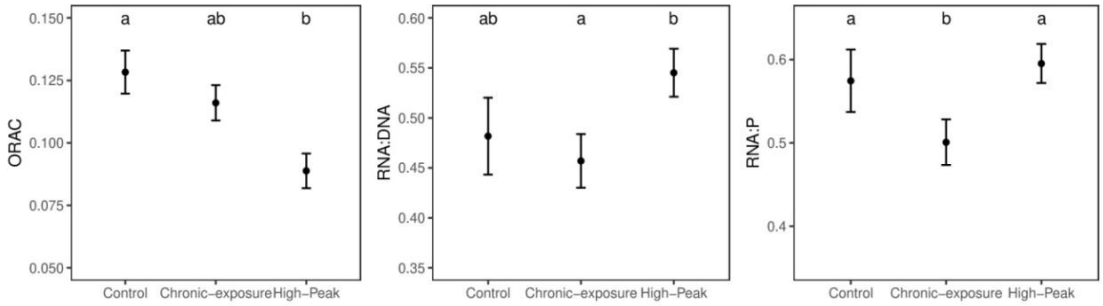

Figure 3. The biomarker responses in *M. affinis* (ORAC, RNA:DNA and RNA:P; mean values and standard errors) significantly affected by the experimental treatments (Control, Chronic exposure and High-Peak). Non-matching letters indicate statistically different groups (Tukey´s test, p < 0.05). See supplementary material D for the responses that were not affected by any of the treatments.

3.3. Correlations among biomarkers

Death rate was negatively correlated with RNA:DNA and RNA:P ratios (p = 0.026 and p = 0.034, respectively; Table 5) as stressed individuals reduced growth rates and increased efficiency of protein synthesis. The negative correlation between the death rate and AChE activity was only marginally significant (p = 0.077; Table 5), indicating that neurotoxicity tended to be associated with lower survivorship. By contrast, death rate was not correlated with TBARS, ORAC and DNA:P (Table 5).

Table 5. Correlation matrix with Pearson´s correlation coefficients between the biomarkers and death rate are shown. Significant correlations (p < 0.05) are in bold. " º " Marginally significant (p < 0.1).

	ORAC	TBARS	AChE	RNA:DNA	RNA:P	DNA:P
Death rate	< - 0.01	- 0.40	- 0.39 º	**- 0.51**	**- 0.55**	- 0.14

No significant cross-correlations between the oxidative status biomarkers (ORAC and TBARS) were observed (Table 6). However, there was a significantly positive correlation between AChE and DNA:P and between AChE and TBARS (p = 0.011; slope = 0.197 and p = 0.039 ; slope = 0.0004, respectively; Table 6). There was also a marginally significant positive correlation between ORAC and RNA:DNA (p = 0.087 ; slope = 0.038, Table 6).

In most cases, the correlations among the biomarkers were consistent for the different treatments (Table 6) as the Treatment:Covariate interaction was not significant. For example, the positive correlation between AChE and DNA:P was

consistent in all three types of incubation (Interaction p = 0.186, Table 6). However, the correlation between ORAC and DNA:P significantly changed between treatments (p = 0.014; Table 6). The correlation showed a positive slope (slope = 0.022; Table 6) in the High-Peak treatment, while it turned to negative in the Control Treatment (slope = - 0.026) and the negative slope became more pronounced in the Chronic exposure treatment (slope = - 0.058). The correlation between AChE and RNA:P was neither consistent between treatments (p = 0.011; Table 6). The negative correlation observed in the Control treatment (slope = - 6.23) was reversed in Chronic exposure individuals (slope = 7.74), becoming more positively pronounced in the High-Peak treatment (slope = 47.60).

4. Discussion

Sediments in aquatic environments are known to act as sinks of a variety of contaminants. Löf et al., (2016a) collected sediment samples along the Bothnian Sea and Bothnian Bay in the Baltic Sea on sites with different pollution levels covering the most representative habitats of *M. affinis*. The observed chemical concentration of the different PCB congeners ranged from $0.03 \cdot 10^{-6}$ to $10 \cdot 10^{-6}$ mg $\cdot$ Kg^{-1} and PAHs (phenanthrene and pyrene) ranged from $0.6 \cdot 10^{-3}$ to $140 \cdot 10^{-3}$ mg $\cdot$ Kg^{-1}. Those concentrations are significantly lower than the ones in the present experiment, however, while 42 toxic compounds were detected in the sampling sites along the Baltic Sea only 7 were used in the present experiment. Additionally, Gorokhova et al., (2010) sampled reference and contaminated sediments of the Baltic Sea (reference sediment: PAH from 0.01 to 0.02 mg $\cdot$ Kg^{-1} and PCB from 0.14 to 0.74 mg $\cdot$ Kg^{-1} and Polluted sediment: PAH from 0.2 to 1.4 mg $\cdot$ Kg^{-1} and PCB from 12.2 to 45.8 mg $\cdot$ Kg^{-1}). They observed that *M. affinis* individuals exposed to contaminated sediments showed signs of chemical stress (i.e., higher lipid peroxidation and neurotoxicity). When comparing chemical concentrations of the present experiment with those described by Gorokhova et al., (2010) we observed that the chemical concentration of the Chronic exposure treatment was between the reference and the contaminated sediment of their study.

Table 6. Significance of the sources of variation when testing for the correlations among variables The effect of Treatment (Control, Chronic exposure and High-Peak), the covariate and covariate:Treatment interaction are shown. Correlations among different nucleic acid ratios were not performed because they were autocorrelated. Complete ANOVA table is shown in supplementary material "E".

Biomarker	Covariate	Treatment p-value	Covariate p- value	Treatment:Covariate p- value
ORAC				
	TBARS	**0.013**	0.484	0.982
	AChE	**0.019**	0.772	0.409
	RNA:DNA	**0.010**	0.087	0.488
	RNA:P	**0.021**	0.823	0.079
	DNA:P	**0.014**	0.112	**0.014**
TBARS				
	AChE	0.980	**0.039**	0.908
	RNA:DNA	0.759	0.501	0.701
	RNA:P	0.654	0.208	0.090
	DNA:P	0.851	0.302	0.068
AChE				
	RNA:DNA	0.903	0.124	0.756
	RNA:P	0.796	0.230	**0.011**
	DNA:P	0.966	**0.011**	0.186

The Chronic exposure treatment presented higher concentrations of PAHs than contaminated sediments whereas lower values of PCBs. The High-Peak treatment concentration was from 4 times higher for PCB 153 to 130 times higher in phenanthrene than contaminated sediments described in Gorokhova et al., (2010). However, Baltic Sea sediments contain a high but unknown number of organic contaminants, which all contribute to the chemical activity of the pollutant mixture on the sediment. Thus, with a mixture of a few compounds as in our study it is difficult to say if we had a similar chemical activity to polluted sites in the Baltic Sea. Chemical activities ranging from 0.01 - 0.1 have been shown to cause 50 % of lethality (Reichenberg & Mayer, 2006; Mayer & Holmstrup, 2008; Smith *et al.*, 2010). In our case, Chronic exposure treatment presented a chemical activity of 0.1 and the High-Peak treatment an activity of 3. The mean CTP of freely dissolved chemicals was 6.4 times higher in the High-Peak treatment compared to the Chronic exposure treatment and therefore, we expected to observe more adverse effects in the High-Peak treatment. Even if several works have reported that chemicals used in this experiment, such as pyrene (Toxværd *et al.*, 2018), phenanthrene (Engraff *et al.*, 2011) or PCB52 (Borgmann, Norwood & Ralph, 1990) affect death rates of exposed individuals, in the present experiment death rate was not affected by any of the treatments. Reutgard and Furuhagen, (2016) performed a laboratory experiment in which *M. affinis* was exposed to pristine and polluted sediments receiving effluent from a pulp mill plant. Even if chemical concentrations present in their polluted sediment were lower than in the present experiment, the incubation time was longer (88-95 days) which made the CTP higher than our Chronic exposure treatment. But, most importantly, at the end of the experiment, survival ranged from 63-77 % and it was not significantly explained by the exposition to the pollutant. The low death rate found in that experiment and in the Chronic exposure treatment of our study suggests that individuals of *M. affinis* are able to withstand high levels of pollution before dying. However, the low death rate observed in the High-Peak treatment was a consequence of the short incubation time. In a parallel 18-d long exposure at high chemical concentration (mean CTP 4.5 fold higher) all individuals died, demonstrating that the high chemical concentration could become lethal if the incubation was long enough.

The biomarkers we selected for this study inform about different physiological processes and are, thus, informative of the status of the amphipods. For instance, the RNA:P ratio indicates efficiency of protein synthesis, as it is proportional to the number of active ribosomes required for the incorporation of one amino acid into the synthesized protein. The reduction of the RNA:P ratio in the Chronic exposure treatment suggests an increase in the efficiency of protein synthesis, which can be linked to the maintenance of the ORAC (oxygen radical absorbance capacity) in this treatment. Exposure to contaminants can lead to both increase and decrease of antioxidant capacity (Livingstone, 2001; Valavanidis *et al.*, 2006); at moderate toxicity individuals might increase their antioxidant capacity to face the stress and when the stress becomes too high, the individuals are not able to match the required antioxidant capacity. Even if an increase in lipid peroxidation could be expected in the presence of the chemical mixture (Giusto *et al.*, 2014; Tucca *et al.*, 2014), the investment in antioxidant defence, indicated by the maintenance of the ORAC, seemed to be enough to avoid increased lipid peroxidation in the Chronic exposure treatment. Individuals exposed to the High-Peak treatment, on the other hand,

significantly increased the RNA:DNA ratio, which suggests an actibation in metabolism. This activation in the metabolism was not observed in the Control and the Chronic exposure treatments. However, even if individuals exposed to a high chemical concentration during 4 days increased their metabolic activity, they were not able to increase the efficiency of protein synthesis and the oxygen radical absorbance capacity significantly decreased. Löf et al., (2016a) observed that ORAC values of *M. affinis* correlated negatively with the concentration of several PCBs, a result which is in line with our observations. In the High Peak treatment the reduction in antioxidant capacity was neither reflected in an increase of lipid peroxidation. This lack of response of lipid peroxidation in the High-Peak treatment could be a consequence of a) the short exposure or, b) the activation of metabolism that could have increased the defence system on *M. affinis*. The AChE is commonly used to detect the neurotoxic stress caused by organophosphates, carbamates and metals (Sarkar *et al.*, 2006). Gauthier et al., (2016) observed that AChE of *Hyalella azteca* exposed to phenanthrene was reduced in a laboratory experiment while Lionetto et al., (2003) observed a reduction of AChE measured on *Mytilus galloprovincialis* from sites exposed to anthropogenic impacts. However, other authors have observed increased AChE activities on individuals to be associated with high levels of oxidative stress (e.g., Wiklund et al., 2014). Contrary to what was expected, in the present experiment the AChE was not affected by any of the treatments. Löf et al., (2016b) observed that while AChE correlated negatively with some PCBs it did show a positive correlation with some PAHs. Therefore, the exposure to a chemical mixture containing both kinds of substances in our study could be behind this lack of reponse from AChE.

The rationale for testing correlations between the biomarkers is based on the fact that we expect to observe certain variability in the response. Animals collected in the field may differ in many traits including gender, age, size, behavior or physiological status (Bolnick *et al.*, 2011), even in stoichiometry (Cai et al. 2016), what can strongly affect their performance under experimental conditions. Thus, the exposure to the same levels of pollution could lead to the animals expressing weaker or stronger responses to the tested impact. On the other hand, as some of the responses of the biomarkers can show maximum/minimum values at intermediate points and others show a monotonic increase or reduction, the correlations among them can change depending on the severity of pollution. With the use of correlations we can more easily pinpoint the degree of toxicity to which a population is being subject to. Death rate was negatively correlated with RNA:DNA and RNA:P ratios, which suggest that stressed individuals reduced their growth rates and shift resources to maintenance by increasing the efficiency of protein synthesis. Moreover, the AChE values showed marginally significant negative correlation with death rate, suggesting that lower survivorship might be associated with higher neurotoxicity. The significant positive correlation between AChE and TBARS observed here was also reported by Reutgard and Furuhagen, (2016). Even if activity of AChE is expected to decrease in response to toxic exposure (Fulton & Key, 2001), it has been suggested that increased AChE activity can be mediated by oxidative stress (Melo, Agostinho & Oliveira, 2003). Therefore, the observed increase in lipid peroxidation would be related to the oxidative stress of exposed individuals. Related to that, the positive correlation between AChE and DNA:P would suggest that individuals with highest oxidative stress (increased AChE) presented the lowest cell division status.

Finally, we observed that individuals with highest metabolic status (RNA:DNA) also presented the highest oxygen radical absorbance capacity (ORAC), which suggest that the high metabolic activity enabled individuals to maintain the oxygen radical absorbance capacity.

The treatments changed only a few of the correlations between biomarkers. Changes in the slope of the correlation were no expected when only considering biomarkers responding monotonically to pollution, such as correlation between lipid peroxidation (TBARS) and neurotoxicity (AChE). On the contrary, when considering correlations for a biomarker with a maximum or a minimum value of the biomarker at intermediate levels of pollution, we expected changes in the sign of the slope. As an example, the correlation between AChE and RNA:P was not the same for the different treatments. We observed that contrary to expected, AChE increased with the oxidative stress and that it correlated positively with lipid peroxidation. The efficiency of protein synthesis (RNA:P) was not significantly different between Control and High-Peak treatments while it was significantly lower in the Chronic exposure treatment, showing the expected hump-shaped response pattern with CTP. As a consequence of this non-linear response, the correlation between RNA:P and AChE changed from being negative in the Control treatment to becoming positive in both chemical exposure treatments (see Fig. 3 for a synthesis of the findings of this study). Thus, as expected, the use of the correlation between variables responding linear and non-linearly to the stressor can be used to improve our understanding of the level of stress in the studied biota. Additionally, the correlation between ORAC and DNA:P was neither constant among treatments; it was negative in Control and Chronic exposure treatments while positive in the High-Peak treatment. ORAC steadily decreased from Control to High-Peak treatment as a consequence of chemical exposure. According to the correlations, the DNA:P increased from Control to Chronic exposure treatment and decreased in the High-Peak treatment. The reduction of the DNA:P ration in the High-Peak treatment could have occurred as a consequence of DNA degradation by ROS accumulation (Halliwell & Gutteridge, 1999). Even if the explanation behind these responses are still unclear, changes in correlations among treatments seems to be a promising tool to more easily assess the level of toxicity the individuals are subject to in the field.

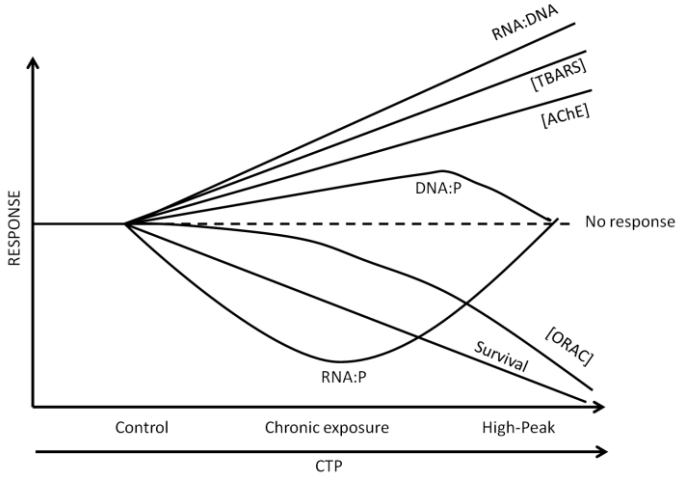

Fig. 3. Conceptual framework modified with the results of the present experiment.

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General conclusions

General conclusions

1- *Echinogammarus berilloni´*s consumption rate was significantly affected by organic matter quality, although the most consumed species (*Fraxinus*) was not the one showing the highest quality (*Alnus*).

2- The quality of the consumed organic matter created subtle effects on amphipod performance. Ingested resource significantly affected amphipod mass body condition but not the rest of the measured variables (death rate, RNA:BM, growth rate and lipid body condition).

3- The observed significant interactions between resource quality and incubation time shows that there are inconsistencies in the response and highlights the importance of standardizing for the incubation time and of increasing the number of replicates that are used in microcosm experiments.

4- Consumption rate and RNA:BM ratio of *E. berilloni* cannot be considered early warning signals in our study because, a) although consumption showed significant responses, it was not followed by changes in amphipod performance during the studied time spam and b) RNA:BM ratio did not respond significantly to the resource quality.

5- After 16 days of incubation amphipod growth rate was significantly responding to the offered resource quality and the internal variability was as low as at day 32. Thus, we recommend a minimum of 16 days of incubation for these kinds of experiments.

6- Supplementing the predominant resource with alder (20 % of the total available resource) only stimulated the consumption rates of the low-quality resources *(Fagus* and *Quercus*), indicating that quality dissimilarities between the available resources are the main forces accelerating consumption.

7- This alder availability created subtle effects on amphipod performance; slight tendencies to increase their growth and, contrary to what was expected, a reduction on amphipod body condition and RNA:BM ratio were observed. Supplementing high quality (*Corylus*) or highly consumed (*Fraxinus*) resources with alder created reductions in body condition, which suggests a cost associated to the selection of the resource.

8- Microbial activity, detritivore consumption and performance did not follow the predicted subsidy-stress response when exposed to the wastewater treatment plant (WWTP) effluent with concentrations ranging from 0 % to 100 %. Microbial respiration and exo-enzymatic activity and detritivore RNA:BM ratio showed a positive relationship with the WWTP effluent concentration.

9- The subsidizing effect of the WWTP effluent for microbial respiration and exo-enzymatic activities was clearer with a longer incubation time.

10- *Monoporeia affinis* individuals exposed to a chronic chemical mixture were not significantly affected in terms of oxidative stress, lipid peroxidation or neurotoxicity.

The lack of response seemed to be related to the increase in efficiency of protein synthesis. The acute toxic scenario significantly reduced the individual oxygen radical absorbance capacity.

11- The highest concentration-time product was the most detrimental for the *Monoporeia affinis* individuals exposed to contaminated sediments.

12- The analysis of the correlations among biomarkers responding linearly and non-linearly to the stressor seemed to assist on the diagnosis of the state of the consumers. However, the use of only three conditions and the need to modify of the initial framework strongly suggest the need for further study.

Future directions

From the results obtained in this PhD, some new questions emerge:

1- Consumption rate and RNA:BM of amphipods fed with different resources could not be established as early warning signals. Which variables could act as early warning signals of the resource quantity and/or quality shortage and precede changes at higher levels of biological organization?

2- We have observed that supplementing other resources with *Alnus* (20 % of total available resource) promoted consumption rates but reduced the body condition, probably due to metabolic cost of actively searching and comparing resources. Which proportion of *Alnus* would deal with this metabolic cost and enable shredders to maintain or increase their body condition?

3- We assessed the effect of a WWTP effluent addition on a field BACI experiment. The measured variables did not significantly respond to the treatment, probably due to high dilution of the effluent. Variables that significantly respond to the effluent in the laboratory microcosm experiment could be used to examine the effect of the WWTP on a large geographic area. What is the degree of dilution and the effect of the effluent on the detrital system in Spain or Europe, for instance? Which percentage of the WWTPs causes subsidizing or stressful effects on receiving stream ecosystems?

4- The effect of the WWTP effluent on streams depends on the dilution factors and the effluent composition. How does seasonality affect the detrital system? Does the lower dilution of the optimally treated effluent in summer affect more than the largely diluted but sub-optimally treated effluent in winter?

5- How would different biomarkers respond along a range of chemical mixture concentration? How would the correlation between linear and non-linearly responding biomarkers change along the chemical mixture concentrations?

Supplementary material

Chapter 1

A- Correlation among leaf traits.

Table SP1. Pearson pairwise correlations between measured leaf litter traits.

Variable 1	Variable 2	t	df	p-value	corr
C:N	C:K	2.23	3	0.112	0.79
C:N	C:P	1.57	3	0.215	0.67
C:N	Toughness	2.26	3	0.109	0.79
C:K	C:P	3.31	3	0.045	0.89
C:K	Toughness	5.18	3	0.014	0.95
C:P	Toughness	5.52	3	0.011	0.95

B- The effect of leaf litter quality.

Table SP2. ANOVA table for the linear models for the response variables including incubation time (Day) and its interaction with Quality as sources of variation.

Source of variation		**Growth rate**		**Mass condition**		**Lipid condition**		**RNA:BM**		**Consumption**	
	DF	*F-Value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
Quality	1	5.33	0.021	0.89	0.345	1.34	0.248	0.57	0.452	139.17	<0.001
Day	4	3.39	0.009	4.04	0.003	0.73	0.572	1.53	0.194	8.48	<0.001
Quality:Day	4	0.80	0.523	6.06	0.0001	0.26	0.901	1.49	0.205	4.31	0.002

Chapter 3

A- Target compounds analyzed in the effluent.

Table SP1. Families, names (abbreviations), supplier of the standards, CAS number, molecular formula, molecular weight, octanol–water partition coefficient ($logK_{OW}$), and acid dissociation constant (pKa) of the target compounds.

Family	Analyte	Supplier	CAS	Molecular	Mw	Log_{KOW}[a]	pKa[a]
Herbicide	Atrazine[b]	Fluka	1912-24-9	$C_8H_{14}ClN_5$	215.68	2.2	3.2
Herbicide	Diuron[b]	Fluka	330-54-1	$C_9H_{10}Cl_2N_2O$	233.09	2.5	13.2
Herbicide	Isoproturon[b]	Fluka	34123-59-6	$C_{12}H_{18}N_20$	206.28	2.6	13.8
Herbicide	Simazine[b]	Fluka	122-34-9	$C_7H_{12}ClN_5$	201.66	1.8	3.2
Hormone	Progesterone	Sigma-Aldrich	57-83-0	$C_{21}H_{30}O_2$	314.46	4.2	-2.6
Hormone	Testosterone	Sigma-Aldrich	58-22-0	$C_{19}H_{28}O_2$	288.42	3.4	-0.9
Industrial chemicals/ Corrosion inhibitor	OBT	Sigma-Aldrich	934-34-9	C_7H_5NOS	151.19	2.5	6.4
Industrial chemicals/ PFASs	PFOSA	Dr. Ehrenstofer	754-91-6	$C_8H_2F_{17}NO_2S$	499.14	4.8	3.4
Industrial chemicals/ PFASs	PFOS[b]	Sigma-Aldrich	1763-23-1	$C_8HF_{17}O_3S$	500.13	5.4	-3.3
Industrial chemicals/ PFASs	PFOA	Sigma-Aldrich	335-67-1	$C_8HF_{15}O_2$	414.07	5.1	-4.2
Industrial chemicals/ PFASs	PFBS	Sigma-Aldrich	29420-49-3	$C_4F_9O_3S$	338.19	2.6	-3.3
Life style products/ Stimulant	Caffeine	Sigma-Aldrich	58-08-2	$C_8H_{10}N_4O_2$	194.19	-0.6	-1.2
Life style products/ Artificial	Acesulfame	Supelco	55589-62-3	$C_4H_5NO_4S$	163.15	-0.6	3.0
Life style products/ Artificial	Sucralose	Supelco	56038-13-2	$C_{12}H_{19}Cl_3O_8$	397.63	-0.5	11.9
PCP/ Preservative	Butylparaben	Sigma-Aldrich	94-26-8	$C_{11}H_{14}O_3$	194.23	3.0	8.5
PCP/ Preservative	Methylparaben	Sigma-Aldrich	99-76-3	C_8H_8O3	152.14	1.7	8.5
Pharmaceuticals/ Antibiotic	Trimethoprim	Fluka	738-70-5	$C_{14}H_{18}N_4O_3$	290.32	1.3	7.2
Pharmaceutical/ Antibiotic	Ciprofloxacin	Fluka	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.34	-0.8	5.7; 8.7
Pharmaceutical/ Antibiotic	Norfloxacin	Fluka	70458-96-7	$C_{16}H_{18}FN_3O_3$	319.33	-0.9	5.7; 8.7

Phamaceutical/ Antibiotic	Sulfadiazine	Sigma-Aldrich	68-35-9	$C_{10}H_{10}N_4O_2S$	250.28	0.4	2.0; 7.0
Phamaceutical/ Antibiotic	Sulfamethoxazole	Fluka	723-46-4	$C_{10}H_{11}N_3O_3S$	253.28	0.8	2.0; 6.2
Pharmaceutical/ Antidepressant	Amitriptyline	Sigma-Aldrich	50-48-6	$C_{20}H_{23}N$	277.40	4.8	9.8
Pharmaceutical/ Antidepressant	Clomipramine	Sigma-Aldrich	17321-77-6	$C_{19}H_{23}ClN_2$	314.85	4.9	9.2
Pharmaceutical/ Antidepressant	Imipramine	Sigma-Aldrich	50-49-7	$C_{19}H_{24}N_2$	280.41	4.3	9.2
Pharmaceutical/ Antidepressant	Nortriptyline	Sigma-Aldrich	72-69-5	$C_{19}H_{21}N$	263.37	4.4	10.5
Pharmaceutical / ARA-IIs	Eprosartan	Solvay Pharma	144143-96-4	$C_{23}H_{24}N_2O_7S$	424.52	3.8	3.6; 6.7
Pharmaceutical / ARA-IIs	Irbesartan	Sanofi	138402-11-6	$C_{25}H_{28}N_6O$	428.53	5.5	4.1; 8.3
Pharmaceutical / ARA-IIs	Losartan	Merck	114798-26-4	$C_{22}H_{23}ClN_6O$	422.91	5.1	3.8; 8.3
Pharmaceutical / ARA-IIs	Telmisartan	Boehringer	144701-48-4	$C_{33}H_{30}N_4O_2$	514.62	6.1	3.6; 4.7; 5.9
Pharmaceutical / ARA-IIs	Valsartan	Boehringer	137862-53-4	$C_{24}H_{29}N_5O_3$	435.52	5.3	4.4; 8.3
Pharmaceutical / β-blocker antihypertensive	Propranolol	MP biomedicals	525-66-6	$C_{16}H_{21}N0_2$	256.34	2.6	9.7;14.1
Pharmaceutical/ Anti-inflammatory	Acetaminophen	Fluka	103-90-2	$C_8H_9NO_2$	151.16	0.9	9.5
Pharmaceutical/ Anti-inflammatory	Diclofenac	Sigma-Aldrich	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	296.15	4.3	4.0
Pharmaceutical/ Anti-inflammatory	Ketoprofen	MP biomedicals	22071-15-4	$C_{16}H_{14}O_3$	254.28	3.6	3.9
Pharmaceutical/ Lipid-regulating	Bezafibrate	MP biomedicals	41859-67-0	$C_{19}H_{20}ClNO_4$	361.80	4.0	3.8
Pharmaceutical/ Lipid-regulating	Clofibric acid	MP biomedicals	882-09-7	$C_{10}H_{11}ClO_3$	214.64	2.9	3.4
Pharmaceutical / Anticonvulsant	Carbamazepine	Sigma-Aldrich	298-46-4	$C_{15}H_{12}N_2O$	236.26	2.8	16.0
Pharmaceutical / Anticonvulsant	Phenytoin	Sigma-Aldrich	57-41-0	$C_{15}H_{12}N_2O_2$	252.20	2.2	8.5
Phytoestrogen	Genistein	Extrasynthese	446-72-0	$C_{15}H_{10}O_5$	270.24	3.1	6.6; 8.0; 9.0
Phytoestrogen	Genistin	Extrasynthese	529-59-9	$C_{21}H_{20}O_{10}$	432.38	0.8	7.3; 9.0;12.5
Phytoestrogen	Glycitin	Extrasynthese	40246-10-4	$C_{22}H_{22}O_{10}$	446.40	1.3	9.0;12.2

a) Values reported in the Free Data Base www.chemicalize.org (16.01.2017)

b) Priority substances.

Abbreviations: ARA-II, angiotensin II receptor; OBT, 2-hydroxybenzothiazole; PFAS, perfluoroalkyl substance; PFBS, nonafluorobutanesulfonate; PFOA; perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PFOSA, perfluoroctylsulfonamide.nonafluorobutanesulfonate; PFOA; perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PFOSA, perfluoroctylsulfonamide.

B- Concentration of the target compounds.

Table SP2. Mean concentrations (ng · L-1) of the target analytes determined by means of active sampling field experiment (Control reach, Impact reach and effluent discharge) during one discharge pulse.

	Impact reach												WWTP effluent discharge					
	0 min	**5 min**	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**	**35 min**	**40 min**	**45 min**	**50 min**	**55 min**	**0 min**	**5 min**	**10 min**	**20 min**	**30 min**	**40 min**
Acesulfame	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acetaminophen	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Amitriptyline	n.d.	24 ±3	25 ±3	24 ±1	24 ±2	27 ±1	26 ±4	34 ±3	25 ±4	13 ±1	< mql	n.d.	71 ±1	69 ±20	74 ±3	72 ±7	73 ±4	75 ±7
Atrazine	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	< mql	< mql	< mql	< mql	< mql	< mql
Bezafibrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	39 ±6	57 ±1	40 ±5	38 ±3	40 ±2	49 ±2	48 ±2	46 ±3	45 ±5	27 ±3	31 ±1	35 ±3	86 ±3	75 ±7	69 ±1	74 ±8	74 ±6	76 ±1
Carbamazepine	n.d.	39 ±1	41 ±2	37 ±2	40 ±1	47 ±1	44 ±2	46 ±1	42 ±2	20 ±1	1.6 ±0.1	< mql	126 ±1	125 ±3	123 ±1	126 ±6	115 ±1	113 ±3
Ciprofloxacin	< mql	45 ±3	42 ±5	37 ±3	46 ±9	49 ±2	43 ±2	43 ±9	45 ±7	23 ±1	22 ±1	< mql	90 ±15	102 ±21	91 ±2	91 ±12	101 ±13	110 ±7
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Clomipramine	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	13 ±2	11 ±3	13 ±1	11 ±1	12 ±1	11 ±1
Diclofenac	n.d.	103 ±20	87 ±2	113 ±2	94 ±17	101 ±25	96 ±19	100 ±22	122 ±20	45 ±2	< mql	n.d.	495 ±10	440 ±103	392 ±74	530 ±101	428 ±8	545 ±148
Diuron	n.d.	85 ±2	88 ±1	92 ±1	91 ±4	108 ±10	103 ±7	118 ±14	110 ±19	39 ±8	25 ±5	17 ±1	277 ±5	270 ±18	271 ±1	278 ±14	256 ±3	247 ±8
Eprosartan	n.d.	141 ±20	141 ±13	123 ±17	194 ±13	262 ±49	185 ±1	174 ±14	174 ±22	81 ±11	< mql	n.d.	632 ±2	683 ±30	610 ±3	782 ±92	746 ±11	694 ±3
Genistein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Genistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycitin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Irbesartan	< mql	1089 ±7	1070 ±36	1019 ±56	1103 ±22	1341 ±85	1250 ±45	1248 ±34	1165 ±10	485 ±26	363 ±23	< mql	3693 ±44	3713 ±2	3609 ±26	3696 ±72	3573 ±26	3547 ±15
Isoproturon	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql
Ketoprofen	n.d.	38 ±9	48 ±12	41 ±6	51 ±9	51 ±10	52 ±11	44 ±2	41 ±3	21 ±1	< mql	n.d.	136 ±9	130 ±6	125 ±17	122 ±12	109 ±3	131 ±16
Losartan	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	12.8 ±0.4	13 ±2	13 ±1	14 ±1	14 ±1	15 ±1
Metylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Norfloxacin	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	34 ±7	33 ±1	31 ±1	38 ±3	34 ±2	34 ±2
Nortriptyline	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	13 ±1	14 ±2	14 ±1	12 ±1	13 ±1	13 ±1
Obt	n.d.	129 ±9	131 ±2	128 ±12	141 ±2	177 ±1	167 ±15	175 ±3	160 ±27	65 ±2	61 ±17	< mql	422 ±11	436 ±35	427 ±0	412 ±21	401 ±3	394 ±22
Pfbs	n.d.	39 ±10	60 ±13	36 ±7	56 ±13	62 ±19	65 ±14	54 ±15	41 ±8	< mql	n.d.	n.d.	141 ±13	173 ±52	162 ±16	135 ±23	131 ±24	143 ±6
Pfoa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pfos	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	n.d.	10 ±2	12 ±1	9 ±1	9 ±2	11 ±1	11 ±1
Pfosa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenytoin	n.d.	536 ±3	513 ±31	490 ±7	523 ±5	593 ±33	596 ±39	587 ±42	551 ±21	214 ±3	167 ±7	< mql	1906 ±1	2042 ±114	1889 ±123	1867 ±110	1830 ±72	1755 ±12
Progesterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	13 ±1	12 ±1	13 ±1	14 ±1	15 ±1	15 ±1	16 ±1	16 ±3	9 ±2	< mql	n.d.	37 ±5	37 ±2	37 ±1	33 ±3	31 ±2	32 ±3
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sucralose	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	1838 ±189	2053 ±526	2119 ±225	1827 ±289	1857 ±324	2170 ±186
Sulfadiazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sulfamethoxazole	n.d.	35 ±1	42 ±3	33 ±4	39 ±1	40 ±5	42 ±1	30 ±1	33 ±1	31 ±12	< mql	< mql	58 ±3	67 ±3	69 ±2	71 ±6	73 ±7	68 ±4
Telmisartan	n.d.	360 ±32	247 ±69	363 ±54	254 ±40	321 ±50	320 ±80	439 ±94	424 ±56	130 ±9	< mql	n.d.	2003 ±454	1535 ±70	1542 ±454	1548 ±364	2021 ±127	1976 ±228
Testosterone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trimethoprim	n.d.	22 ±2	24 ±2	22 ±1	24 ±4	27 ±2	26 ±1	28 ±3	26 ±4	15 ±2	16 ±3	1.5 ±0.5	41 ±8	45 ±4	47 ±4	45 ±4	41 ±2	42 ±2
Valsartan	n.d.	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	< mql	n.d.	327 ±4	368 ±94	339 ±21	418 ±80	466 ±17	501 ±10

n.d. non detected; < mql below its limit of quantification, a WWTP effluent dischar lasts 40 minutes.

C- Medium M9 adapted from Sambrook and Russel D. (2001)

Na_2HPO_4 .....6.0 g · L^{-1}
KH_2PO_4 ..... 3.0 g · L^{-1}
NH_4Cl .....1.0 g · L^{-1}
NaCl .....0.5 g · L^{-1}
$MgSO_4 \cdot 7H_2O$ solution .....1.0 mL· L^{-1}
$CaCl_2$ solution.....1.0 mL· L^{-1}

pH 7.0 ± 0.2 at 25°C

$MgSO_4 \cdot 7H2O$ Solution: 24.65 g $MgSO_4$ + 100 mL H_2O

$CaCl_2$ Solution: 1.47 g $CaCl_2$ + 100 mL H_2O

D- Emergent pollutant analysis procedure

Active and passive sampling analysis.

A solid phase extraction (SPE) method previously validated (Mijangos *et al.*, 2018a) was used for the extraction of the samples. Briefly, an appropriate volume of a sodium ethylenediaminetetraacetic (Na_2EDTA) solution to achieve a final concentration of 0.1 % (g solute/g solution) was added and samples were acidified (pH = 2) with formic acid prior to the extraction. Oasis HLB 200 mg-cartridges were sequentially conditioned with 5 mL of Methanol (MeOH), 5 mL of Milli-Q water and 5 mL of Milli-Q water at pH = 2. The sample (100 mL in the case of effluent and 250 mL in the case of estuary) was, then, percolated through the cartridge assisted by a vacuum pump at ca. 5 mL/min. Subsequently, the cartridges were washed with 6 mL of ultrapure water, vacuum dried for 40 min and eluted with 6 mL of MeOH. After elution, the extract was concentrated to dryness under a gentle stream of nitrogen at 35 C and reconstituted in 200 µL of MeOH: Milli-Q water (30:70, v:v). Finally, the reconstituted extracts were filtered through a 0.2 µm PP filter before the LC–MS/MS analysis.

Regarding the passive sampling, POCIS were prepared according to the procedure described previously (Mijangos *et al.*, 2018b) Briefly, POCIS sorbent was carefully removed from the membranes using approx. 10 mL of Milli-Q water and introduced into empty SPE cartridges. The sorbent was dried under vacuum for ~ 1 h, and then, eluted with 6 mL of MeOH with 2.5 % ammonium followed by 6 mL of MeOH. The mixture was evaporated to dryness using a TurboVap LV Evaporator at 35 ^{0}C and reconstituted in 200 µL of MeOH: Milli-Q (30:70, v:v) mixture. Finally, the extracts were filtered through a 0.22 µm PP.

LC-MS/MS analysis.

Analysis were carried out using a HPLC-QqQ (Agilent 1260 series LC coupled to an Agilent 6430 triple quadrupole) equipped with electrospray ionization (ESI) source (Agilent Technologies) according to a previously optimized method (Mijangos *et al.*, 2018a).

The separation of the target analytes was accomplished at a flow of 0.3 mL/min using a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 µm) column coupled to a Kinetex F5 pre-column (2.1 mm x 4.6 mm, 2.6 µm). The column temperature and the injection volume were set to 35

°C and 5 μL, respectively. Under optimized conditions, a binary mixture consisting on a mobile phase A of water: MeOH (95: 5) and mobile phase B of MeOH: water (95: 5), both containing 0.1 % of formic acid was used for gradient separation of the target analytes. The gradient profile started with 30 % B which was increased to 50 % in 4 min and maintained for 12 min. Then it was increased to 90 % B where it was maintained constant for 10 min. Initial gradient conditions (30 % B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). ESI was carried out using a nitrogen flow rate of 12 L/min, a capillary voltage of 3500 V, a nebulizer pressure of 45 psi, and a source temperature of 350 ºC. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection.

Quantification was performed in the selected reaction monitoring (SRM) acquisition mode. Instrumental operations, data acquisition and peak integration were performed with the MassHunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

Chapter 4

A- Chemical mixture used in the experiment

All solvents used (dimethylformamide from VWR chemicals; acetone and hexane from Merck) were of HPLC grade. PCBs ($^{13}C_{12}$) were from Wellington Laboratories, and HCB ($^{13}C_6$) from Cambridge Isotope Laboratories. Deuterated PAHs (d_{10}) were from Ladoran Fine Chemicals AB, and native PCB_3 used as an internal surrogate standard from Fluka Analytical. Native CB_{53} and aldrin used as internal recovery standards, as well as PCBs, PAHs and HCB used for sediment spiking were from Accustandard.

Chemical concentrations were calculated based on the estimated total organic carbon content of the food (45 %). Spiked concentrations: 1350 mg · kg^{-1} HCB and biphenyl and 900 mg · kg^{-1} PCBs and PAHs for the High-Peak treatment or two orders of magnitude less for the Low-Long treatment. Fish flakes were spiked by adding chemicals in stock solutions (3 mg · ml^{-1} HCB dissolved in hexane; 3 mg · ml^{-1} biphenyl, 2 mg · ml^{-1} PCBs and 2 mg · ml^{-1} PAHs dissolved in acetone) on top of the dry fish flakes, after which the solvents were evaporated.

Sediment samples were air dried and extracted with accelerated solvent extraction (ASE, Dionex 200). Dry sediment (0.1 g) was added to 34 ml extraction cells, thereafter internal standard (IS) surrogate standards (CB3, $pyrene_{D10}$, $phenanthrene_{D10}$, HCB_{C13}) were added, after which the cells were filled with ISOLUTE® HM-N resin (furnished 450 ºC overnight prior use). For quality assurance, method blanks for which the cells were filled with only ISOLUTE® HM-N resin. Acetone:hexane (1:1 v:v) was used for extraction, with two extraction cycles per sample. Instrument setup followed (Josefsson *et al.*, 2006). Sample extracts were treated with activated Cu powder for sulfur removal. Due to the high expected concentrations in the extracts, the samples were diluted (1:20 Low-Long and 1:400 High-Peak), after which a second batch of IS surrogate standards were added ($CB52_{C13}$, $CB101_{C13}$, $CB153_{C13}$). The cleanup of the samples was done with a DMF method, after which the samples were passed through a silica (10 % MilliQ Water w:w, SiO_2 - gel 60 Merck) column (1 cm ø), topped with Na_2SO_4 (VWR chemicals). The clean up method is described in detail in Mustajärvi et al. (2017).

Equilibrium passive samplers were used to determine C_{free}. Sediment samples for C_{free} analysis were collected at the end of the experiment (day 22) and frozen until analysis. Passive equilibrium sampling was carried out by using glass jars coated internally with a 1, 3 or 5 µm silicone layer (DC1-2577- silicone; Dow Corning®) (Reichenberg *et al.*, 2008). Approximately 10 g of wet sediment was added to the coated 20 ml glass jars. The glass jars were rolled horizontally for two weeks in room temperature in darkness, to allow the analytes to equilibrate between the sediment and the silicone coated on the inner walls of the glass jars. The sediment was then discarded and the inside part of the glass vials was rinsed with cold tap water and dried carefully with a lint-free tissue. For extraction of the silicone, 2 ml acetone:hexane (1:1, v:v) was added to the glass jars together with IS surrogate standards (CB3, $pyrene_{D10}$, $phenanthrene_{D10}$, HCB_{C13}). The glass jars were rolled for 30 minutes and solvent was collected. Due to the high expected concentrations in silicone, the samples were diluted after extraction (1:10 Low-Long and 1:400 High-Peak). The extraction of the silicone was repeated for a second time and the two extracts were pooled.The cleanup of the extracts followed the same procedure as described for the sediment samples. After cleanup all samples were reduced in volume (200

µl) and recovery standards (PCB53 and Aldrin) were added prior the gas chromatography mass spectrometry (GCMS) analysis.

Chemical Analysis Quality assurance

Limit of detection (LOD) was calculated as the average amount detected in the blank samples plus three times standard deviation, and all samples were blank corrected. Average blank levels for equilibrium passive sampler (n = 8) were 0.90 ± 1.03 ng for HCB, 0.21 ± 0.25 ng for PAHs and 0.61 ± 0.21 ng for PCBs. The average blank levels for sediment samples collected prior to the experiment were 2.06 ± 0.09 ng, 2.69 ± 0.41 ng and 2.17 ± 1.36 ng, and at the end of the experiment 0.34 ± 0.00 ng, 1.03 ± 0.41 ng and 0.46 ± 0.07 ng respectively. The average recoveries of the internal standards for C_{free} analyses were 83 ± 36 % HCB, 64 ± 11 % PAHs and 59 ± 13 % PCB. Average recoveries for the sediment samples prior to the experiment were 45 ± 7 %, 66 ± 12 % and 68 ± 13 %, and after experiment 28 ± 10 %, 51 ± 17 % and 54 ± 10 % respectively. In all cases, the chromatographic peaks were easily identified and the internal standards were used to correct losses during extraction and cleanup. No consistent differences were observed between the internal standard recoveries added to the samples prior (HCB_{C13}, $phenanthrene_{D10}$, $pyrene_{D10}$ and CB_3), and after dilution ($CB52_{C13}$, $CB101_{C13}$, and $CB153_{C13}$), indicating that dilution was not causing a major problem in the analysis.

During the 12 days of the spiking process (equilibrating the chemical mixture sorbed to the inside if the glass jars and sediment), the concentration of some chemicals was reduced. Losses were correlated with the compound octanol-water partition coefficient (log K_{ow}). Hence, the compounds with higher K_{ow}, such as the PCBs (log K_{ow} 6.09 - 7.16), were better retained in the sediment. Despite the high spiking concentration, biphenyl (log K_{ow} 4.01) was entirely lost during the spiking process (see Table X). In the Low-Long treatment, after the spiking process, sediment presented 36 % and 68 % of the nominal spiked concentrations for HCB and phenanthrene. Pyrene and PCB concentrations measured in sediment after spiking corresponded well to the nominal spiked concentrations (95 – 133 %). In the High-Peak treatment, losses of chemical concentration during the spiking process were higher than in the Low-Long treatment. In the High-Peak treatment, 6 % of the nominal concentration was retained in the sediment after the spiking process for phenanthrene and 20 %, 23 % and 60 % for HCB, pyrene and PCBs, respectively.

B- Concentration-time product in sediment concentrations and freely dissolved concentrations.

Table SP1. Sediment measured concentrations (mg · Kg^{-1}), CTP of each chemical compound and CTP ratio between Chronic exposure treatment and High-Peak treatment (CTP ratio).

	Chronic exposure concentration	Chronic exposure CTP	High-Peak concentration	High-Peak CTP	CTP ratio
HCB	3.10	68	169	676	9.94
Biphenyl	n.d.	-	n.d.	-	-
PCB 52	3.55	78	252	1008	12.92
PCB 101	3.79	83	240.5	962	11.6
PCB 153	3.00	66	181.4	726	11
Pyrene	1.63	36	108	432	12
Phenanthrene	0.60	13	26.53	106	8.15

n.d.: No detected.

Table SP2. Freely dissolved concentrations (mg · L^{-1}), CTP of each chemical compound and CTP ratio between Chronic exposure treatment and High-Peak treatment (CTP ratio).

	Chronic exposure concentration	Chronic exposure CTP	High-Peak concentration	High-Peak CTP	CTP ratio
HCB	0.00126	0.02772	0.00797	0.03188	1.15
PCB 52	0.00215	0.0473	0.12263	0.49052	10.37
PCB 101	0.00006	0.00132	0.00243	0.00972	7.36
PCB 153	0.00002	0.00044	0.00042	0.00168	3.82
Pyrene	0.00281	0.06182	0.14482	0.57928	9.37

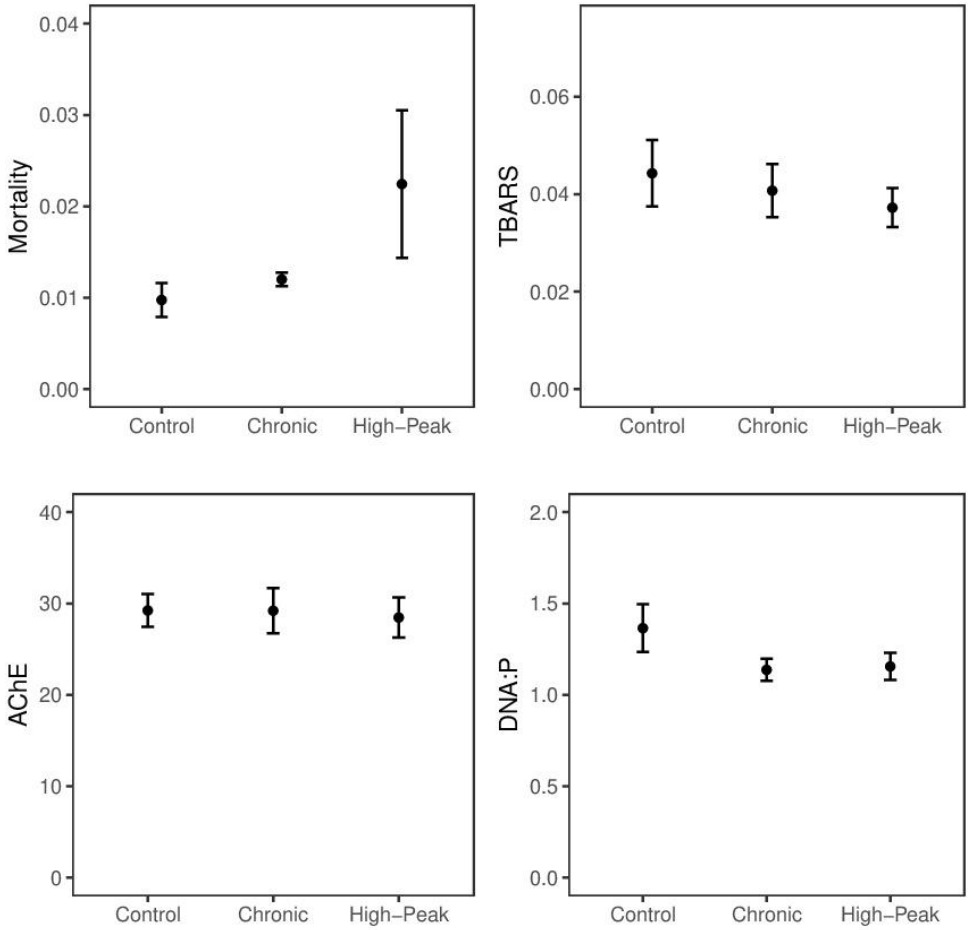
C- The effect of incubation time on Control individuals biomarkers.

Table SP3. Statistical results for differences between individuals at the beginning (T0) and individuals collected after 22 days of incubation in the Control treatment.

Biomarker	ss	ms	NumDF	DenDF	*F-value*	*P-value*
ORAC	0.004	0.002	2	3.81	1.31	0.368
TBARS	0.0049	0.0025	2	3.60	0.75	0.534
AChE	347,81	173.91	2	2.16	2.00	0.322
RNA:DNA	0.093	0.046	2	2.49	1.19	0.433
RNA:P	0.024	0.012	2	41	0.58	0.562
DNA:P	0.1567	0.078	2	2.36	1.85	0.329

D- Non-significant responses among the treatments for *M. affinis*

Figure SP1. *M. affinis* response variables (Mortality, TBARS, AChE and DNA:P) not significantly affected by the different treatments (Control, High-Peak and Chronic exposure). Mean values and standard errors are represented.



E- Complete ANOVA table for the linear models built for the relationship among biomarkers.

Table SP4. Significance of the sources of variation when testing for the correlations among variables. The effect of Treatment (Control, Chronic exposure and High-Peak), the covariable and covariable:Treatment interaction are shown. Significant p values are highlighted in bold.

Biomarker	**Covariable**	**Treatment**						**Covariable**						**Treatment:Covariable**					
		ss	Num DF	Den DF	ms	*F-value*	*p-value*	ss	Num DF	Den DF	ms	*F-value*	*p-value*	ss	Num DF	Den DF	ms	*F-value*	*p-value*
ORAC																			
	TBARS	0.0135	2	10.20	0.0068	4.73	**0.013**	0.0003	1	74.39	0.0003	0.21	0.484	0.0001	2	74.36	0.00003	0.023	0.982
	AChE	0.01199	2	12.31	0.0059	4.19	**0.019**	0.00001	1	80.26	0.00001	0.01	0.772	0.0023	2	79.89	0.00116	0.81	0.409
	RNA:DNA	0.0121	2	14.21	0.0060	4.45	**0.010**	0.0021	1	74.57	0.0021	1.56	0.087	0.0018	2	74.43	0.0008	0.65	0.488
	RNA:P	0.01168	2	14.27	0.0058	4.38	**0.021**	0.00005	1	76.43	0.00005	0.04	0.823	0.0068	2	76.31	0.0033	2.48	0.079
	DNA:P	0.0116	2	13.73	0.0058	4.67	**0.014**	0.0039	1	77.49	0.0039	3.16	0.112	0.0102	2	76.24	0.005	4.11	**0.014**
TBARS																			
	AChE	0.00002	2	8.67	0.00002	0.03	0.980	0.0014	1	72.38	0.0014	3.05	**0.039**	0.0001	2	69.95	0.00005	0.11	0.908
	RNA:DNA	0.00017	2	10.83	$8 \cdot e^{-5}$	0.16	0.759	0.0001	1	68.50	$7 \cdot e^{-5}$	0.13	0.501	0.0003	2	68.58	$1.7 \cdot e^{-4}$	0.32	0.701
	RNA:P	0.013	2	11.74	0.0067	0.83	0.654	0.029	1	70.54	0.029	3.6	0.208	0.035	2	70.52	0.017	2.18	0.090
	DNA:P	0.0003	2	9.33	0.0001	0.29	0.851	0.002	1	69.87	0.002	3.9	0.302	0.0026	2	70.22	0.0013	2.57	0.068
AChE																			
	RNA:DNA	0.039	2	77	0.019	0.27	0.903	0.1627	1	77	0.1627	2.3	0.124	0.013	2	77	0.0067	0.09	0.756
	RNA:P	95.87	2	78	47.94	0.41	0.796	467.85	1	78	467.84	4.05	0.230	1013.27	2	78	506.64	4.38	**0.011**
	DNA:P	0.009	2	76	0.005	0.07	0.966	0.403	1	76	0.403	6.08	**0.011**	0.211	2	76	0.105	1.59	0.186