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TESIS DOCTORAL

ESTUDIO ECOTOXICOLÓGICO DE LOS EFECTOS DE AGROQUÍMICOS EN LA ESTRUCTURA Y EL FUNCIONAMIENTO DE LA COMUNIDAD PLACTÓNICA

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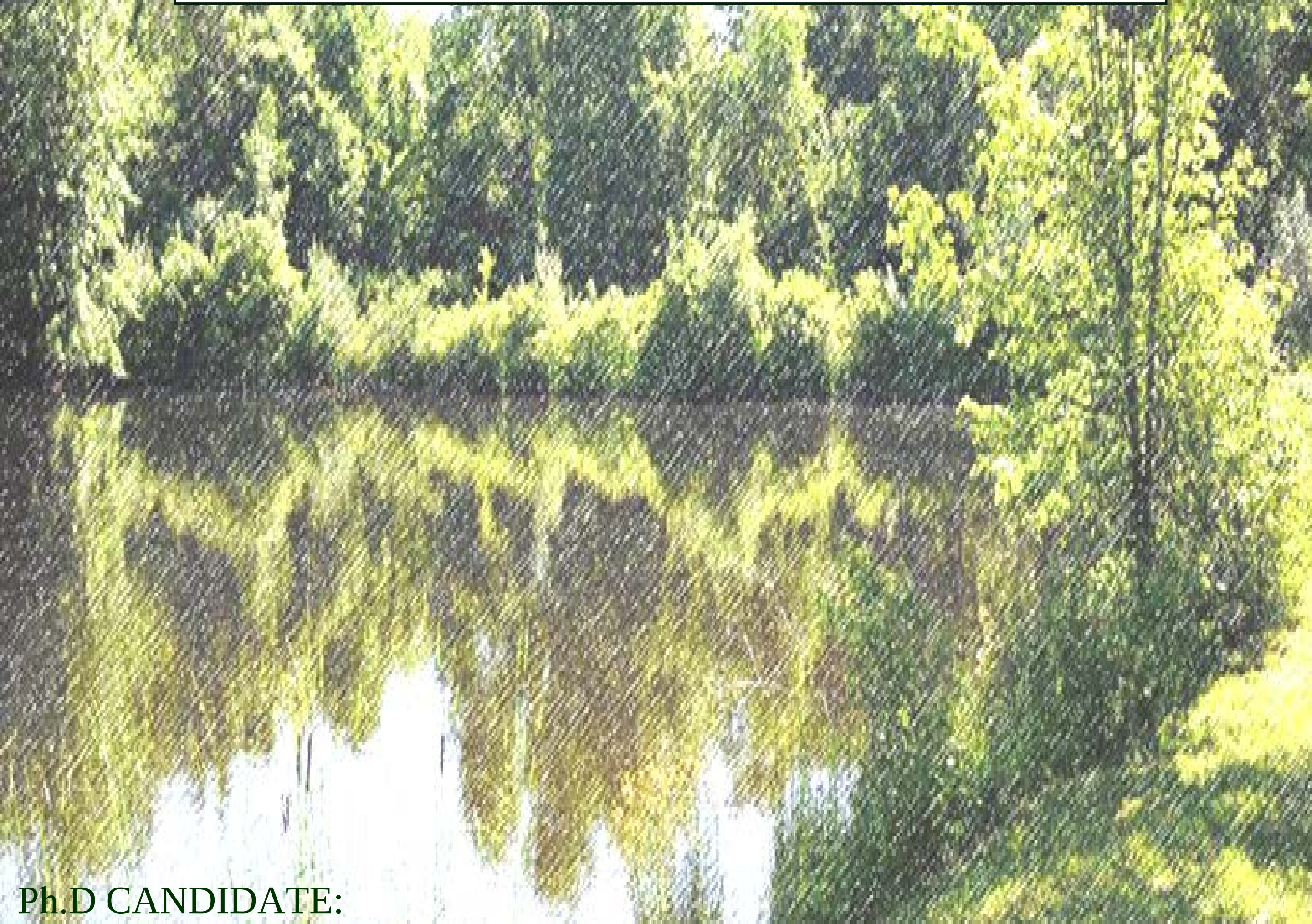
ECOTOXICOLOGICAL STUDY OF AGROCHEMICALS' EFFECTS ON PLANKTON COMMUNITY STRUCTURE AND FUNCTION



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ANA ISABEL DEL ARCO OCHOA

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Departamento de Biología Animal, Biología Vegetal y Ecología

Área de Ecología

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Tesis Doctoral

Ana Isabel Del Arco Ochoa

Jaén 2015

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ECOTOXICOLOGICAL STUDY OF AGROCHEMICALS' EFFECTS ON PLANKTON COMMUNITY STRUCTURE AND FUNCTION.

Memoria para optar al grado de Doctor con Mención Europea por la Universidad de Jaén

Presentada por:

Ana Isabel Del Arco Ochoa

Esta tesis ha sido realizada bajo la dirección de

Vº Bº Directora

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CERTIFICA

Que la presente memoria, titulada "Estudio ecotoxicológico de los efectos de agroquímicos en la estructura y el funcionamiento de la comunidad planctónica", ha sido realizada bajo mi dirección y presenta, a mi juicio, contenido científico suficiente, por lo que autorizo su presentación y defensa para poder optar al grado de Doctor en la modalidad de Doctorado con Mención International por la Universidad de Jaén por cumplir los requisitios exigidos por la normativa para tal fin.

Jaén, diciembre de 2014

Fdo.: Mª Gema Parra Anguita

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A mi hermano Samuel por su humor y “otros motivos” ☺

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ABSTRACT

Wetlands importance for global cycles and theirs ecosystem services as biodiversity have been internationally recognized. Despite such recognition wetlands contamination and disappearance occurs. One of the pressures on wetlands comes from agriculture because of the need of land for crops resulting in its drainage, contamination, silting, transformation in irrigation ponds and eutrophization. Human population growth and its effects on agricultural needs and in higher agrochemical use will result in higher wetland pollution. European policy efforts are being made to achieve good chemical and ecological quality of all European water bodies. In order to prevent ecological impairment, one of the tools is the Ecological Risk Assessment (ERA) of chemical. The ERA evaluates the effect of toxicant exposures upon ecosystems, animals and humans. There is an objective asymmetry between community protection policy goals and ERA ecological realisms since it is mainly based on single-species tests. Therefore, an improvement of ERA is needed in order to increase its ecological relevance to facilitate management decisions. The needed areas to balance the mentioned objective asymmetry, which have been highlighted by ecotoxicology experts and European agencies, are: 1) influence of ecological interactions as competition and 2) predation on community responses and recovery capacity after toxicant exposures; 3) explore toxicant effects on biodiversity and 4) on ecosystem services and functions; and, 5) assess toxicant mixtures effects. Therefore, the thesis focuses on agrochemical scenarios (mixture, pulses frequency and limits) and ecological scenarios (ecological interactions as competition and hierarchical levels) through six chapters. There are three main objectives within those scenarios: 1) Assess the effect of agrochemicals commonly used above and below legal limits on aquatic community in order to test if current legislation over- or under protect aquatic communities; 2) Assess how agrochemicals mixture and pulses frequency effects on aquatic community vary compare to single agrochemical exposures; and 3) Assess the influence of ecological interactions on the sensitivity response of aquatic species to agrochemicals. The working hypothesis under this scenarios and objectives is that agrochemicals exposure due to prevailing agriculture intensive practices has negative effects on the aquatic community integrity at both structural and functional levels. As expected, negative effects of agrochemicals on plankton community were found despite of agrochemical concentrations being within legal limits. Moreover, the thesis deals with mixtures and frequency of agrochemicals exposures what seeks to simulate more realistic field chemical exposures. Results show a higher effect of single agrochemical exposures than mixture exposures, which is explained by indirect effects that counterbalance for direct toxic effects across the trophic web of one of the chemicals within this specific mixture. However, other results shown no compensation effects in mixtures versus single exposure because a drastic toxic effect (due to the insecticide) hiding potential interaction at lower mixtures concentrations. In addition to mixture, treatment frequency was not relevant because has been also hidden for the drastic effect of the insecticide since the first application. The ecological interactions play a role in the sensitivity of aquatic organisms; the thesis regards the assessment of the effects of intra- and interspecific competition on macroinvertebrates exposed to a fungicide. However, it is complex to predict the positive or negative influence because it will vary depending on diverse factors as species, density pressures and behavioral aspects. The complexity of the results analysis shows how complex are natural systems, it stimulates scientific creativity for future research projects.

RESUMEN

La importancia de los humedales en los ciclos globales y de sus servicios ecosistémicos, como la biodiversidad, ha sido reconocida internacionalmente. A pesar de dicho reconocimiento, la contaminación y desaparición de humedales sigue teniendo lugar. Una de las actividades que genera presiones sobre los humedales es la agricultura, principalmente por la necesidad de terrero para cultivos, lo que conlleva su desecación, contaminación, colmatación, transformación en balsas de regadío y eutrofización. El crecimiento de la población y el incremento en necesidades agrícolas junto a un mayor uso de agroquímicos da lugar a una mayor contaminación de los humedales. Las políticas europeas se están esforzando en conseguir una buena calidad química y ecológica de todos los sistemas acuáticos europeos. Con el objetivo de prevenir un deterioro ecológico, uno de las herramientas es la Evaluación del Riesgo Ecológico (ERA) de productos químicos. La ERA evalúa los efectos de los tóxicos sobre los ecosistemas, los animales y los humanos. Sin embargo, hay una asimetría entre las metas políticas de protección de las comunidades y el realismo ecológico de ERA debido a que se basa principalmente en los resultados de los tests toxicológicos con una única especie. Por tanto, se necesita una mejora del procedimiento de ERA para aumentar su relevancia ecológica y facilitar las decisiones de gestión. Las áreas necesarias para equilibrar dicha asimetría, las cuales han sido destacadas por expertos en ecotoxicología y agencias europeas, son: 1) la influencia de interacciones ecológicas como la competencia y 2) la depredación en la respuesta de la comunidad y en su capacidad de recuperación tras la exposición a químicos; 3) explorar los efectos de los tóxicos en la biodiversidad y 4) en los servicios ecosistémicos y en sus funciones; y, 5) evaluar los efectos de mezclas de tóxicos. En consecuencia, la tesis se centra en escenarios agroquímicos (mezclas, frecuencia de pulsos y límites) y escenarios ecológicos (interacciones ecológicas como la competencia y niveles jerárquicos) a través de seis capítulos. Hay tres objetivos principales dentro de los mencionados escenarios: 1) Evaluar los efectos de agroquímicos comúnmente usado tanto por encima como por debajo de límites legales en las comunidades acuáticas con lo que se pretende comprobar si los límites actuales de legislación protegen o no a las comunidades acuáticas; 2) Evaluar cómo los efectos de la mezcla de agroquímicos y la frecuencia de pulsos en las comunidades acuáticas varía en comparación con la exposición a un único compuesto; y 3) Evaluar la influencia de las interacciones ecológicas en la respuesta de sensibilidad de las especies acuáticas a la exposición de agroquímicos. La hipótesis de trabajo bajo estos escenarios y objetivos es que la exposición a agroquímicos debido a las prácticas de agricultura intensiva predominantes tiene efectos negativos sobre la integridad de la comunidad acuática tanto a niveles estructurales como funcionales. Como se esperaba, se detectaron efectos negativos de agroquímicos en la comunidad planctónica incluso bajo concentraciones de agroquímicos dentro de los límites legales. Además, la tesis trata la mezcla y la frecuencia de la exposición de los agroquímicos lo cual aspira a simular exposiciones químicas más realistas y aproximarse a las condiciones de campo. Algunos de los resultados obtenidos muestras efectos mayores tras la exposición a un único compuesto en comparación con una exposición donde hay mezcla de agroquímicos, lo que se explica debido a efectos indirectos que implican una compensación de los efectos directos a través de la red trófica de uno de los químicos dentro de las mezclas específicas bajo estudio. Sin embargo, otros resultados no muestras efectos de compensación en mezclas *versus* exposiciones bajo un único compuesto (insecticida), lo que podría ocultar interacciones potenciales de mezclas de menor concentración. Sumado a las mezclas, la frecuencia del tratamiento no fue relevante posiblemente porque también pueda estar oculta debido a los efectos drásticos del insecticida desde la primera aplicación. Las interacciones ecológicas desempeñan un papel importante en la sensibilidad de los organismos acuáticos; la tesis considera la evaluación de los efectos de la competencia intra- e interespecífica en macroinvertebrados bajo la exposición de un fungicida. Sin embargo, es complejo predecir si la influencia es positiva o negativa porque ésta varía dependiendo en diversos factores como las especies, las presiones de densidad y aspectos del comportamiento. La complejidad en el análisis de resultados muestra en sí misma la propia complejidad de los sistemas naturales, a la vez que estimula la creatividad científica para la planificación de investigaciones futuras.

INTRODUCTION

Wetlands, the threatened aquatic ecosystems

Aquatic ecosystem embrace water bodies from large scale, as the oceans, to small scale, as ponds, being both scales equally important for global cycles and biodiversity (Downing, 2010). Within aquatic ecosystems, wetlands have been characterized by high economic, cultural, recreational, educational, and scenic values, characterized by high productivity and habitat heterogeneity, which results in a large landscape diversity and biodiversity (Mitsch and Gosselink, 2000). Considering the Ramsar definition, wetlands are “areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six meters” (Ramsar, 1971). The important of wetlands have been internationally recognized from the Convention on *Wetlands* (Ramsar, 1971) to nowadays (Millennium Ecosystem Assessment, 2005). Wetlands provides, regulate and support diverse ecosystem services as drinkable water, water storage, flooding control, nutrients cycling, biodiversity, migratory habitats among others (Millennium Ecosystem Assessment, 2005). Despite theirs values and international efforts to protect then wetland contamination and disappearance is a reality. The present study has taken place in the context of Spain, a Mediterranean country where more than 60% of wetlands have disappeared (Casado and Montes, 1995); even when the wetlands are maintained, they are suffering many anthropogenic impacts (Ortega *et al.*, 2006). More specifically, this thesis has been developed in Andalucía which is rich in Mediterranean temporary ponds (MTPs). MTPs are considered high eco-social values for its peculiarity since are found in only 5 regions in the world with Mediterranean climate (Rhazi *et al.*, 2006), therefore, are a priority habitat in European networks as Natura 2000. Going a local step further, in the Alto Guadalquivir area (Jaén and eastern Córdoba region) there are more than 90 wetlands and just 14 of them are protected. The situation is worrying, actually one has been drained despite its protection (Ortega *et al.*, 2003) what raise concern about the other wetlands future.

Wetlands and agriculture

Wetlands have faced a conflict with agriculture land needs meaning its drainage, pesticides and herbicides contamination, silting, transformation in irrigation ponds and eutrophization (Ortega *et al.*, 2003). In fact, it has been reported that wetlands surrounded by agriculture are one of the most stressed natural systems due to agricultural development and runoff (Casado and Montes, 1995; Salvadó *et al.*, 2006; Schulz *et al.*, 2003). A future worst case scenario would be expected because human population growth will lead to an increase of agricultural needs worldwide so then to higher agrochemical use. Therefore, more use of fertilizers and pesticides as fungicides and insecticides that will impact aquatic systems. For instance, the European Environmental Agency have already published a fertilizer use increase of 35% from 138 million ton in 1999 to 188 million in 2030 if the current intensive and inefficient management practices continue (EEA, 2014). In addition, global changes represent a multiple stressor scenario where warmer and more humid conditions are expected (Harvell *et al.*, 2002). It may lead to an increase of insect and fungi resulting in a higher pest occurrence, consequently ending in a higher use of agrochemical potentially reaching aquatic systems. That situation will have an impact at social and economic levels owing to the loss of ecosystems services like a key one as biodiversity. Anthropic activities impact on biodiversity at both terrestrial and freshwater ecosystems, however this impact have been reported to be more drastic in aquatic systems (Sala *et al.*, 2000; Ferreira, 2008). Indeed, agrochemicals negative effects on aquatic systems lead to higher adverse consequences on terrestrial biodiversity (García-Muñoz *et al.*, 2010, 2011).

European policy efforts are being made to prevent wetlands pollution. European authorities are aware of these ecological risks and make efforts to prevent, reduce and mitigate then. For instance, the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) of the European Union (EU) had marked the need to further investigate chemical effects on aquatic organisms. Nonetheless, a balance between agriculture development and environmental protection is complex to achieve due to diverse agendas at economic and ecological levels sum up to the involvement of diverse actors (Kaika, 2003). In this sense, it is worthy to mention a specific example that link *a priori* positive politic agricultural decision with negative environmental consequences. The Common Agriculture Policy (CAP) pretended to provide a positive economic and social incentive to rural areas with low crop production (classified as Less Favored Areas (LFA)). However, the final results have been an inefficient and dependent agriculture on subsidies in this LFA together with environmental degradation (Caraveli, 2000). Figure 1a shows the European map of LFA (OECD, 1997), it is worthy to give particular attention to the fact that in South Spain the LFA areas coincide with the Alto Guadalquivir (Figure 1b) where 80% of wetlands are impacted by agricultural practices (Ortega *et al.*, 2003). Considering our immediate environment, Andalucía is characterized by the olive tree agriculture. In fact, Spain is the world's leading olive groves producers and Andalucía accounts for more than 60% of the Spanish olive cultivated area and 32% of the EU (Junta de Andalucía, 2014a). In social terms, it means that olive groves are the main economic activity in more than 300 villages supporting more than 250.000 families (Junta de Andalucía, 2014b). It represents a complex scenario between environmental and economic criteria. To embrace agriculture development and environmental protection entails complex challenges hard to harmonize. However, environmental relevance to maintain economic welfare cannot be underestimated as has been the case in many decisions in the past. Hence, this thesis seeks to contribute to the balance between agrochemical use in agriculture and aquatic ecosystems protection. For this purpose, the data presented pretend to support the increasing voices claiming more ecologically realistic risk assessments (ERA).

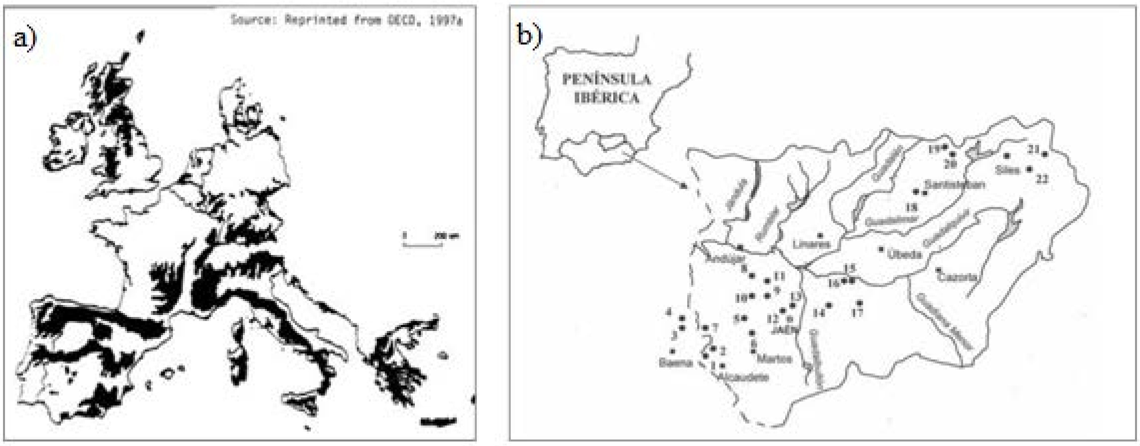

Figure 1. a) Less-favored areas: uplands; b) Alto Guadalquivir wetlands (Ortega *et al.*, 2006).

Thesis framework

The pressure-state-response (PSR) framework states that human activities exert *pressures* on the environment, which can induce changes in the *state* of the environment. Then society has to develop *responses* to changes in pressures or state with environmental and economic policies and programs to prevent, reduce or mitigate pressures and/or environmental damage (Rodríguez, 2010). The PSR framework has been considered a good conceptual tool for both identify the thesis context and its clearness for public communication (Figure 2).

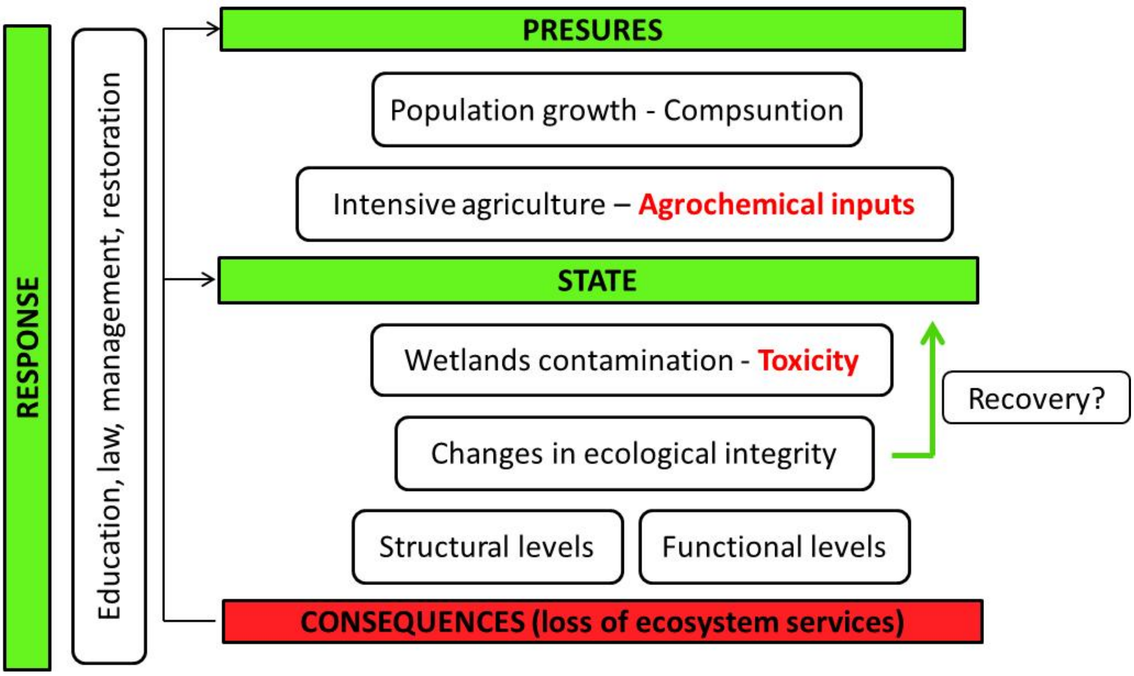

Figure 2.Conceptual model of the thesis framework.

Therefore, this thesis focuses on one of the agricultural pressures upon water bodies: agrochemicals inputs. As agrochemicals generate changes in the environmental state through their toxicity, the study was considered into Ecotoxicology. Ecotoxicology is “a subdiscipline of ecology that focuses on the effect of toxic substances on ecosystems and their living components” (Jorgensen, 2010). Ecotoxicological studies are helping to understand individuals, population and community responses to chemical exposures giving science-based evidences to take decisions. In this sense, European Union efforts walk towards a good chemical and ecological quality of all European water bodies by 2015 (Directive 2000/60/CE). One of the tools to evaluate chemicals impacts on the ecosystems is the Ecological Risk Assessment of chemicals (ERA).

ERA is a set of different techniques and methodologies (Figure 3) to examine the effect of toxicant exposures on ecosystems, animals and humans (EEA, 2014). Ecotoxicology is mainly related and devoted to the ecological effects assessment. Frequently, the effects characterization and assessment is based on PNEC calculation (Predicted No-Effect Concentration) that indicates the level of exposure that does not produce adverse effects on ecosystems. The easiest way to calculate PNEC is using the quotient method comparing toxicity to environmental exposure. In this method, the estimated environmental concentration (EEC) is compared to an effect level, such as an LC50 (the concentration of a toxic substance where 50% of the organisms die). When the quotient is bigger than 1, toxic risk exists. Initially, agrochemicals thresholds have been based on laboratory toxicity test to assess toxicant exposure effect based on endpoints (survival, growth or reproduction) in single species test and single compound, such as the classical LC50. But, two main shortcomings are associated with this approach: a) the extrapolation of the effect based on endpoint at individual levels into a complex ecosystem context with populations and communities (Van den Brink, 2013; Wootton, 2002; Brooks *et al.*, 2009), and b) chemicals do not occur alone in the environment, consequently mixtures should also be studied to better understand direct and indirect effect of agrochemicals inputs (LeBlanc *et al.*, 2012; Anderson *et al.*, 2006; Barata, 2006).

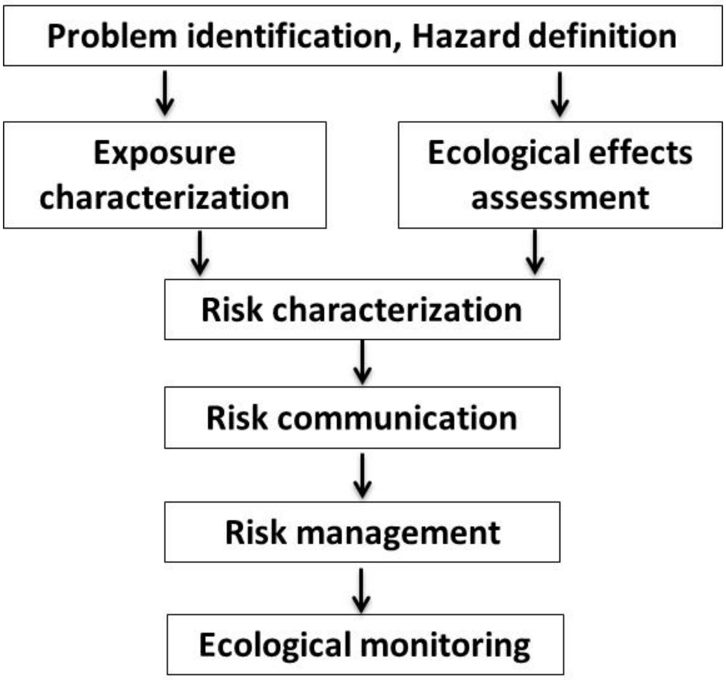

Figure 3.The conceptual diagram for ecological risk assessment (ERA) together with risk communication, management and ecological monitoring (Jorgensen, 2010).

Organizations as the Organization for Economic Co-operation and Development (OECD), the World Health Organization (WHO) and the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) are actively working on the improvement of ERA in order to facilitate management decisions (EEA, 2014). In fact, it is informally known that EU directives protection goals based on individual-levels would need to increase its complexity level in order to fulfill population and ecosystem protection goals (De Laender *et al.,* 2013). De Laender *et al.* (2013) highlight 5 main study areas to balance that objective asymmetry: study the influence of 1) competition and 2) predation on population and community levels and recovery capacity; 3) explore the chemical effects on biodiversity; 4) assess chemical exposure on ecosystem services and functions; and, 5) evaluate toxicant mixture effects. ERA seeks to establish standards for the use of chemicals to reduce ecological risk. In order to do so, ERA may have to be updated considering the 5 main study areas mentioned above in order to stablish protective enough legal limits. In this respect, current legal limits are based on single species tests which lack ecological and chemical realisms. Therefore, it would be insufficient to obtain a realistic ecological risk assessment where direct and indirect effect would be difficult to evaluate (Crane, 1997; Boxall *et al.*, 2002; Baird *et al.*, 2007). All the mentioned weaknesses represent an intricate challenge where it must be accepted that: a) it is extremely complicated to assume the cost of all environmental effects and, b) environmental managers have to take decision dealing with high uncertainty (Figure 4; Jorgensen, 2010). The aptitude to deal with this high complexity is not to get discouraged about the possibility of taking action to prevent environmental impacts. On the contrary, the consequence is to invest in research to take more science-based decision and to apply the precautionary principle when there is lack of information.

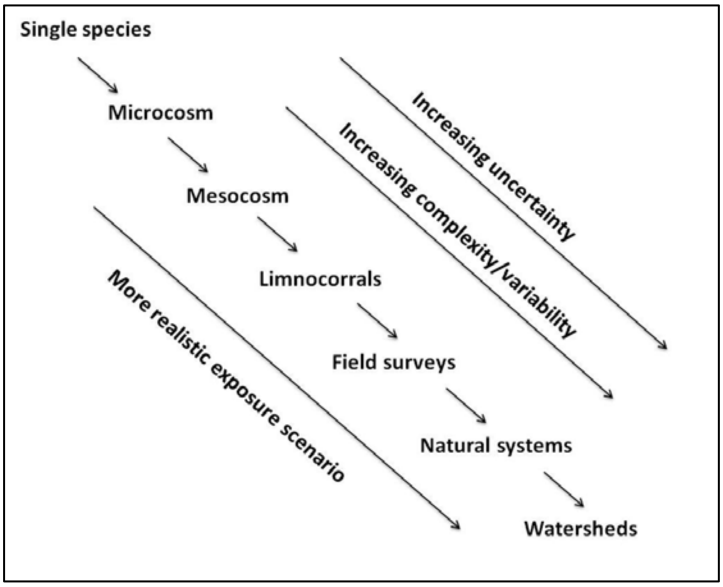

Figure 4. Aquatic ecotoxicological tests used to establish biologically safe concentrations of potential toxicants (Modified from Jorgensen, 2010). The diagram shows the difficulty of combine complex ecological studies with interpretation confidence.

Thesis scenarios

From this unexplored new scientific niche, this thesis explores scenarios of chemical mixtures, of ecological conditions and of theirs interactions. Reassuming, the first rule to deal with complex scenarios is to simplify. In order to contribute to a better understanding of agrochemical effects on the aquatic communities, this work encompasses two main scenarios through 6 chapters: agrochemical scenarios (mixture, pulses frequency and limits) and ecological scenarios (ecological interactions: competition and hierarchical levels) (Figure 5).

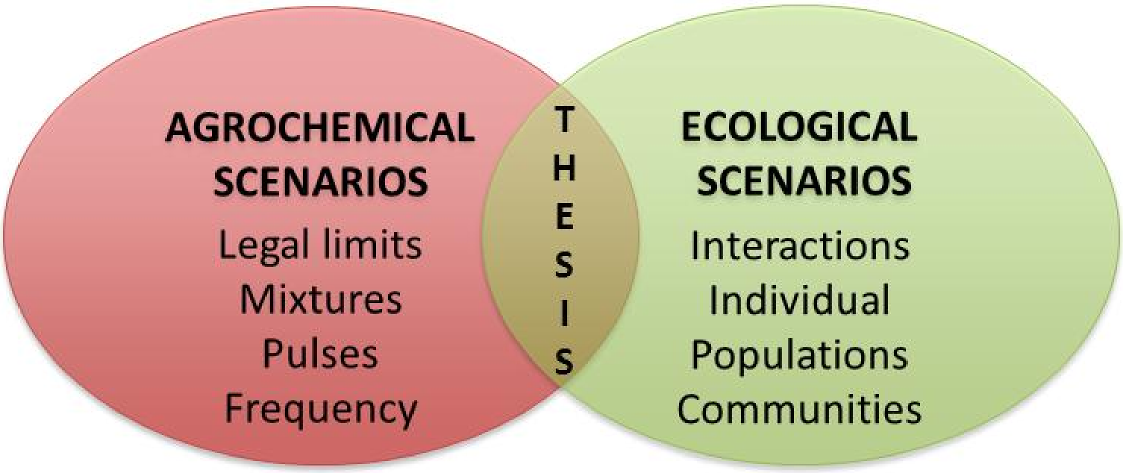

Figure 5. Two main scenarios of the thesis.

The first scope deals with the complexity of agrochemicals exposure scenarios in the environment that mainly depend on the agriculture uses and legal limits. Chemical concentrations in runoff events will vary with seasonality and application times. In addition, there will be mixtures of the chemicals applied together in the same season and/or with the ones already present in the environment from previous applications. Therefore, the prevalence of interactive toxic effects over the occurrence of single solutions is a fact that should be studied (Kungolos *et al.*, 2009). Such interactive effect could be: additive when chemicals do not interact and the effect is the sum of theirs toxicity effect; synergetic when chemicals interact and its combined effect is higher than if additive behavior would have occurs; and, antagonistic when chemical interact and the toxicity effect of one counterbalance the effect of the other chemical. In addition, a further relevant branch is to explore the low doses of chemicals routinely detected in the environment (LeBlanc *et al.*, 2012) that may fall within legal limits but still affecting aquatic systems. Further than this thesis focus, it raises the question if sublethal routinely detected levels of contamination influence microevolution or local adaptations towards more tolerant species.

Agrochemicals used in agriculture will reach aquatic ecosystems by both direct spray and runoff events (Brock *et al.*, 2006). The mode of action (pesticides, fungicides, fertilizers…) and the chemical characteristics (sequestration, detoxification, bioaccumulation, synergy…) have repercussion on the final toxic effect and could increase the ecological risk. One section of the experiments regards toxicity modulation, considering toxicant concentrations below and above legal limits, in order to detect ecological risk. The use of concentrations above legal limits is justified because pollutants concentrations can increase up to several orders of magnitude after rainfall events (Rabiet *et al.*, 2010). The interest in study toxicant concentrations below legal limits comes from the potential sublethal effect of low doses routinely detected in the environment (Maltby *et al.*, 2002; LeBlanc *et al.*, 2012). For instance, one consequence of sublethal effect could be the development of tolerance of specific life stages or species what ultimately means a deviation from unpolluted communities. Brausch and Salice (2011) reported that the second generation of *Daphnia magna* was not affected by a low environmental realistic pesticides concentration what suggests the development of tolerance. Therefore, toxicant concentrations below legal limits may have sublethal effect that may be critical to better understand effects on field populations and communities. Apart from the toxicant concentration, pulse addition is also a relevant factor that is becoming more important in ecotoxicological studies. Most of the studies assess constant toxicant concentrations (Hecnar, 1995; García-Muñoz *et al.*, 2009), however, pulses addition of the same concentration could have different effects (Earl and Whiteman, 2009; García-Muñoz *et al.*, 2011). In field conditions, contaminant exposures of aquatic organisms fluctuate in concentration, duration and frequency (Hoang *et al.*, 2007; Downing *et al.*, 2008). Agrochemicals inputs in aquatic systems will vary mainly owing to: runoff related to rainfall events; and, application timing depending on the cultivated species growth requirements and control of pests and diseases (FAO, 2006; LeBlanc *et al.*, 2012; Haygarth *et al.*, 2012; Reinert *et al.*, 2002; Hoang *et al.*, 2007; Earl and Witheman, 2009). For that reason, experiments presented in this thesis explore both single and several pulses. Other section of the experiments deals with responses to single and mixture toxicant exposure. For instance, in Delta del Ebro more than 30 pesticides are routinely detected in surface water (Suárez-Serrano *et al.*, 2010), however, pesticides effects on aquatic organisms are based on individual toxicant (Lydy *et al.*, 2004). It is obvious the high complexity of extrapolate Ecological Risk Assessment (ERA) to field conditions when considering a single toxicant. Therefore, even if considering mixtures would be more appropriate, the complexity exponentially increases. Nevertheless, studies of mixtures are needed in order to fulfill such as relevant knowledge gap about complex exposure scenarios. In fact, the European Commission highlight that only few semi-field experiments assessed mixtures of pesticides effects on aquatic organisms and ecosystem functions (European Commission, 2006). This thesis explores the toxic effects of fertilizers (ammonium nitrate, nitrogen and phosphorus), fungicides (copper sulfate and carbendazim) and insecticide (chlorpyrifos). Agrochemicals selection responds to different criteria. Ammoniun nitrate and copper sulfate have been chosen because of its local relevance in olive tree groves in Jaén (Andalucía) likewise its global use in other cultures. Experiments have focus con nitrate because ammonium quickly transform into nitrates by nitrification processes. Even though nitrate naturally occurs in aquatic systems, its concentrations have increased due to intensive fertilizers use. García-Muñoz *et al.* (2011) reported effects on amphibian tadpoles (*Epidalea calamita*) mortality and total length of sublethal pulses of nitrate. In the case of copper sulfate, it can even been directly applied into water systems as a plant herbicides and algaecide around the world. In addition, copper is a heavy metal what is a major category of pollutants impacting also human health (Duruibe *et al.,* 2007).Copper sulfate is the commercial product used in agriculture, but copper is the target chemical from the experiments. Even though copper could be found naturally in different forms, it can be toxic in aquatic systems as Cu^{2+} (Lenwood *et al.*, 1998). Previous studies have report direct effect on individuals and long-term effects on populations. Parra *et al.* (2005) showed copper effect on hatching rates and nauplii survival on the copepod *Arctodiaptomus salinus*; while, Johnston and Keough (2005) reported changes in population size structure of sessile marine invertebrates as a results of copper pulses.

Nutrients (nitrogen and phosphorus) and chlorpyrifos are the agrochemicals used to explore mixture, pulses and pulses frequency scenarios. Nitrogen cycle has been highly disrupted by human activities meaning highly inputs in aquatic systems through runoff events from agricultural fields, livestock and atmospheric deposition (Galloway *et al.*, 2004). Phosphorus cycle has also been altered by human activity increasing its release from the sediments (Mooij *et al.*, 2005) or entering by runoff events from fertilization cropping season, consequently altering natural aquatic systems. For instance, Miracle *et al.* (2007) proposed a relationship between nutrients inputs in aquatic ecosystems and a shift to a turbid water state as a result of zooplankton community change nutrient-induced. Nutrients changes co-occur with other chemical pressures as insecticide pollution. Chlorpyrifos is a broad-spectrum organophosphate insecticide used for agricultural purposes worldwide. Brock *et al.* (2000) reported insecticides toxicological effects upon growth, survival and reproduction of aquatic organisms. Chlorphyrifos and nutrients are likely to occur in the aquatic systems due to its application times ending in runoff events (FAO, 2001; FAO, 2006; Reinert *et al.*, 2002). Moreover, Carbendazim is a worldwide fungicide to control pest in oilseed rape, maize and rice among others cultures. Carbendazim negative effect on macroinvertebrate species (Cuppen *et al.*, 2000) and zooplankton species (Van den Brink *et al.*, 2000) has been previously described. The different types of agrochemicals have shown negatives effects on different organisms but also they could affect higher hierarchical levels such as community structure changes. Examples of some studies encompassing community complexity are Van den Brink *et al.* (2000) and Downing *et al.* (2008). Van den Brink *et al.* (2000) assessed the effects of a fungicide (carbendazim) in zooplankton and primary producers. They found structural changes as a consequence of both direct toxicant adverse effects on zooplankters and macroinvertebrates, and indirect effects of phytoplankton growth owing to grazing pressure release. In the same line, Downing *et al.* (2008) studied the freshwater community responses to environmental realistic pesticide (Sevin) pulses finding decreases of zooplankton endpoints (richness, diversity, abundance and oxygen concentrations) while increases in phytoplankton and microbial endpoints (abundance).

The second scope focuses on the ecological conditions. Ecological risk assessment use single standard species test which results are used to stablish legal limits of chemicals. However, a main shortcoming of those tests is the lack of ecological relevance where ecological interactions are ignored and only standards species instead of local species are used. Hence, the mentioned weakness compromises results extrapolation into ecosystem levels. The aquatic community is the thesis “studied subject”. It is known that pesticides and fertilizers can impact the ecological integrity of the aquatic community. The changes in its structure or function can be used as endpoint to assess the toxic effects at this hierarchical level. Traditionally, effects at morphological, physiological, biochemical or genetic levels have been reported (Troncoso *et al.*, 2000). Consequently, species recruitment, hatching rates, survival rates, grazing capacity will be alter (Parra *et al.*, 2005; Sharp and Stearns, 1997) raising concern about long term consequences for the ecosystems and the services they provide. In this sense, field experiments have linked algae blooms events with a decrease of invertebrate grazers affected by insecticides (Hurlbert *et al.*, 1972, Boyle *et al.*, 1996). Algae blooms could lead to eutrophization problems changing water quality, submerge vegetation density and biodiversity (Miracle *et al.*, 2007) consequently, diminishing ecosystem services. The effects of agrochemicals on aquatic systems have been regulated with a focus on standard species (for instance, *Daphnia* sp. or *Chironomus* sp. for invertebrates, green algae for algae or *Lemna sp*. for macrophytes). However, agrochemicals will affect local communities that most likely differ from the few standard species better studied. In order to overcome this limitation microcosms experiments were done using natural local communities’ assemblages. The microcosms allowed establishing natural biological assemblages to assess toxicant exposure at population and community levels (Figure 4). Multispecies studies are not always available even though they have the potential to provide more ecological realistic data than single species test. Microcosms brings the possibility of assess indirect effects, recovery capacity and structural-functional relationships (OECD, 2006; Sanderson *et al.*, 2009). Therefore, microcosms stablished with co-existing population in natural local wetlands were considered a proper set up for the goals of the thesis. In addition, cosmos with a lower level of complexity were also use in order to explore specific ecological interaction as competition. The use of simple ecological interactions studies in combination with community experiments may help to identify mechanisms controlling direct and indirect community responses to agrochemical exposure. This combination of experimental studies would allow a holistic effect interpretation at higher scales of complexity. In this context, it is crucial to consider biological and ecological factors as species present, genotype, life stage and ecological interactions (Hanazato *et al.*, 2001; De Laender, 2013). Ecological interactions are a recent research area recommended to be considered in Ecological Risk Assessments (Van den Brink, 2013; De Laender *et al.*, 2013; Brocks *et al.*, 2006). It will contribute to not underestimate or overestimate agrochemical risk for the aquatic communities (Pestana *et al.*, 2009; Foit *et al.*, 2012).

The emerging recognition of the role of small wetlands ecosystems (i.e. small lakes and ponds as are the majority of Jaén wetlands) in global processes and cycles being this research area mostly unexplored (Downing *et al.*, 2010) is the mayor justification for the present work. In these aquatic ecosystems, Plankton (phytoplankton and zooplankton) is an important component at ecosystem levels for being a principal pathway for energy flow (Álvarez Cobelas and Rojo, 2000; Nayar *et al.*, 2004). Phytoplankton is the primary producer in aquatic systems, therefore, its community structure and dynamic influence higher trophic levels as zooplankton. Changes in phytoplankton community could result in inedible taxa for zooplankton communities. Reduced grazing capacity of zooplankton are of extreme importance because can result in eutrophication impacts (Van Wijngaarden *et al.*, 2005; Hanazato, 1998; Fleeger *et al.*, 2003). Zooplankton represents one of the major components of lake ecosystems having an influence on the water quality and upon other trophic levels. Previous studies highlight the consequences of zooplankton abundance and community changes upon the spring clear-water phase in lakes (Hanazato, 1998) and on fish larvae development (Zagarese, 1991). Benthos taxa were also used, the taxa selection criteria were based on its ecological importance likewise other relevant features for the experimental design as its natural co-occurrence and easy handle characteristic. Then, competition experiments were conducted using gasteropods (*Bithynia tentaculata* and *Radix peregra*), amphipods (*Gammarus pulex*) and isopods (*Asellus aquatic*). In the case of gastropods, its ecological relevance is due to its high abundance in aquatic systems up to 20%-60% of the biomass of macroinvertebrates in some freshwater ecosystems (Habdija *et al.*, 1995). And, amphipods are considered major decomposer of leaf material what is a crucial ecosystem function (Zubrod *et al.*, 2010; Graça et al., 1994).

Based on the previous sections and paragraphs, we defend the need of this research, conceptual framework, scenarios, methodology, likewise the agrochemical selection and species used. It supports the following hypothesis and objetives.

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INTRODUCCIÓN

Los humedales, sistemas acuáticos amenazados

Los sistemas acuáticos engloban cuerpos de agua desde aquellos de gran escala, como océanos, hasta los de pequeña escala, como charcas, siendo ambas escalas igual de importantes para los ciclos globales y la biodiversidad (Downing, 2010). Dentro de los sistemas acuáticos, los humedales han sido reconocidos por su alto valor económico, cultural, recreativo, educativo y escénico. Su alta productividad y heterogeneidad de hábitats da lugar una extensa diversidad de paisajes y alta biodiversidad (Mitsch and Gosselink, 2000). Según la definición en Ramsar, los humedales son "áreas de marismas, pantanos y turberas, o agua, tanto natural como artificial, permanente o temporal, estática o corriente, dulce, salobre o salada, incluyendo áreas de agua marina cuya profundidad en marea baja no supera los seis metros" (Ramsar, 1971). La importancia de los humedales ha sido internacionalmente reconocida desde la Convención sobre Humedales (Ramsar, 1971) hasta la actualidad (Millennium Ecosystem Assessment, 2005). Los humedales aprovisionan, regulan y sustentan diversos servicios de los ecosistemas como de almacenamiento y abastecimeinto de agua, control de inundaciones, ciclo de los nutrientes, biodiversidad, habitar migratorios entre otros (Millennium Ecosystem Assessment, 2005). A pesar de sus valores y de los esfuerzos internacionales para protegerlos, la contaminación de humedales y su desaparición es una realidad. El estudio que se presenta tiene lugar en el contexto de España, un país mediterráneo donde más del 60% de los humedales han desaparecido (Casado and Montes, 1995); incluso donde los humedales persisten, estos están bajo numerosos impactos antrópicos (Ortega *et al.*, 2006). Más específicamente, esta tesis se ha desarrollado en Andalucía la cual es rica en Humedales Temporales Mediterráneos (en inglés Mediterranean Temporary Ponds, MTP). Los MTP se consideran sistemas de alto valor ecológico y social por su peculiaridad, ya que sólo se encuentran en 5 regiones con clima Mediterráneo en el mundo (Rhazi *et al*., 2009), por tanto, es un hábitat prioritario a proteger en redes Europeas como Natura 2000. Desde un enfoque aún más local, en el Alto Guadalquivir (Jaén y la zona este de Córdoba) hay más de 90 humedales y tan sólo 14 de ellos están protegidos. La situación es cuanto menos preocupante, máxime cuando uno de ellos ha sido secado a pesar de estar protegido (Ortega *et al.*, 2003), lo cual genera una preocupación aún mayor sobre el futuro del resto de humedales.

Humedales y agricultura

Los humedales han estado supeditados a las necesidades de la producción agrícola, lo que ha dado lugar a su desecación, contaminación con pesticidas y herbicidas, colmatación, transformación en balsas de regadío y eutrofización (Ortega *et al.*, 2003). De hecho, hay información que constata que los humedales rodeados de zonas agrícolas son uno de los sistemas naturales más alterados debido al desarrollo de la agricultura intensiva junto al aumento de la escorrentía (Casado and Montes, 1995; Salvadó *et al.*, 2006; Schulz *et al.*, 2003). Un escenario aún peor se espera porque el crecimiento de la población humana llevará a un aumento de las necesidades agrícolas a nivel mundial, por tanto también a un uso mayor de agroquímicos, lo que impactará de forma negativa a los sistemas acuáticos. Por ejemplo, la Agencia Europea de Medio ambiente (AEMA) ha publicado que habrá un aumento de fertilizantes del 35% pasando de 138 millones de toneladas en 1999 a 188 millones de toneladas en 2030 si continúan las prácticas agrícolas intensivas e ineficientes de la actualidad (EEA, 2014). Además, los cambios globales representan un escenario de presiones múltiples donde se prevén condiciones climáticas más cálidas y húmedas (Harvell *et al.*, 2002). Esto puede conllevar un aumento de las poblaciones de insectos y hongos que resultaría en una mayor incidencia de plagas, consecuentemente provocando un aumento del uso de agroquímicos que potencialmente podrían llegar a los sistemas acuáticos. Dicha situación tendrá, a su vez, impacto a niveles sociales y económicos debido a la pérdida de servicios de los ecosistemas, tales como la biodiversidad. Las actividades antrópicas impactan la diversidad tanto de sistemas terrestres como acuáticos, sin embargo se ha defendido que los impactos son más drásticos en sistemas acuáticos (Sala *et al.*, 2000; Ferreira, 2008). De hecho, los impactos negativos de los agroquímicos en los sistemas acuáticos repercuten a su vez negativamente en la diversidad de los sistemas terrestres (García-Muñoz *et al.*, 2010, 2011). La unión europea está haciendo esfuerzos políticos para prevenir la contaminación de los humedales. Las autoridades europeas son conscientes de los riesgos medioambientales y apuestan por su prevención, reducción o mitigación. Por ejemplo, el Registro, Evaluación, Autorización y Restricción de químicos (REACH) de la EU ha destacado la necesidad de investigar en profundidad los efectos de sustancias químicas en organismos acuáticos. No obstante, es complejo alcanzar un equilibrio entre el desarrollo agrícola y la protección del medio ambiente debido a sus diferentes agendas e intereses a nivel económico y ecológico sumado a la implicación de agentes muy diversos (Kaika, 2003). En este sentido, merece la pena mencionar un ejemplo que enlaza una decisión política positiva *a priori* en el sector agrícola que acabó siendo una consecuencia medioambiental negativa. La Política Agraria Común (PAC) pretendía proporcionar un incentivo positivo social y económico a zonas rurales con poca producción (clasificadas como zonas desfavorecidas). Sin embargo, el resultado ha sido una agricultura ineficiente y dependiente de subsidios in dichas áreas desfavorecidas ligada a una degradación medioambiental (Caraveli, 2000). La figura 1a muestra un mapa de las zonas europeas clasificadas como desfavorecidas (OECD, 1997), merece la pena prestar atención al hecho de que en el Sur de España parte de las áreas desfavorecidas coinciden con el Alto Gualquivir (Figura 1b) donde el 80% de los humedales presentan distintos niveles de alteración debido a las prácticas agrícolas (Ortega *et al.*, 2003). Si se considera nuestro entorno más inmediato, Andalucía se caracteriza por el cultivo del olivar. De hecho, España es el líder mundial en producción del olivar y Andalucía representa más del 60% de las zonas españolas con olivar y un 32% de la EU (Junta de Andalucía, 2014a). En términos sociales, esto significa que el olivar es la principal actividad económica in más de 300 pueblos donde más de 250.000 familias dependen de ésta (Junta de Andalucía, 2014b). Esta situación representa un escenario complejo entre criterios medioambientales y económicos. Equilibrar la protección agrícola y medioambiental conlleva retos complejos difíciles de armonizar. Sin embargo, no se puede desestimar la importancia medioambiental para mantener un bienestar económico como ha ocurrido en el pasado durante los procesos de toma de decisiones. Por lo tanto, esta tesis persigue contribuir al balance entre el uso de agroquímicos en agricultura y la protección del medio acuático. Con dicho objetivo, los datos presentados pretenden apoyar el aumento de voces pidiendo evaluaciones de riesgo ambiental más realistas ecológicamente.

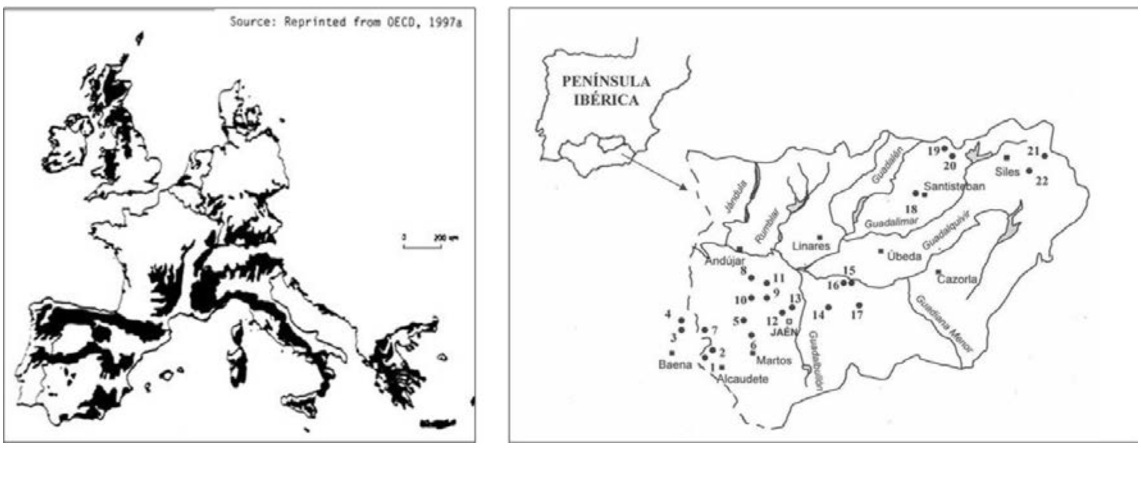

Figura 1. a) Zonas desfavorecidas: b) Humedales del Alto Guadalquivir (Ortega *et al.*, 2006).

Marco conceptual

El marco conceptual de Presiones-Estado-Respuestas (PER) establece que la actividad humana ejerce *presiones* sobre el medio ambiente, lo cual induce cambios en su *estado*. En consecuencia la sociedad tiene que desarrollar *respuestas* a cada cambio de presión o estado a través de políticas medioambientales y económicas así como programas para prevenir, reducir o mitigar las presiones y daños medioambientales (OECD, 2003; Rodriguez, 2010). El marco conceptual PER se ha considerado una buena herramienta conceptual tanto para presentar el contexto de esta tesis como por su claridad para una comunicación pública (Figura 2).

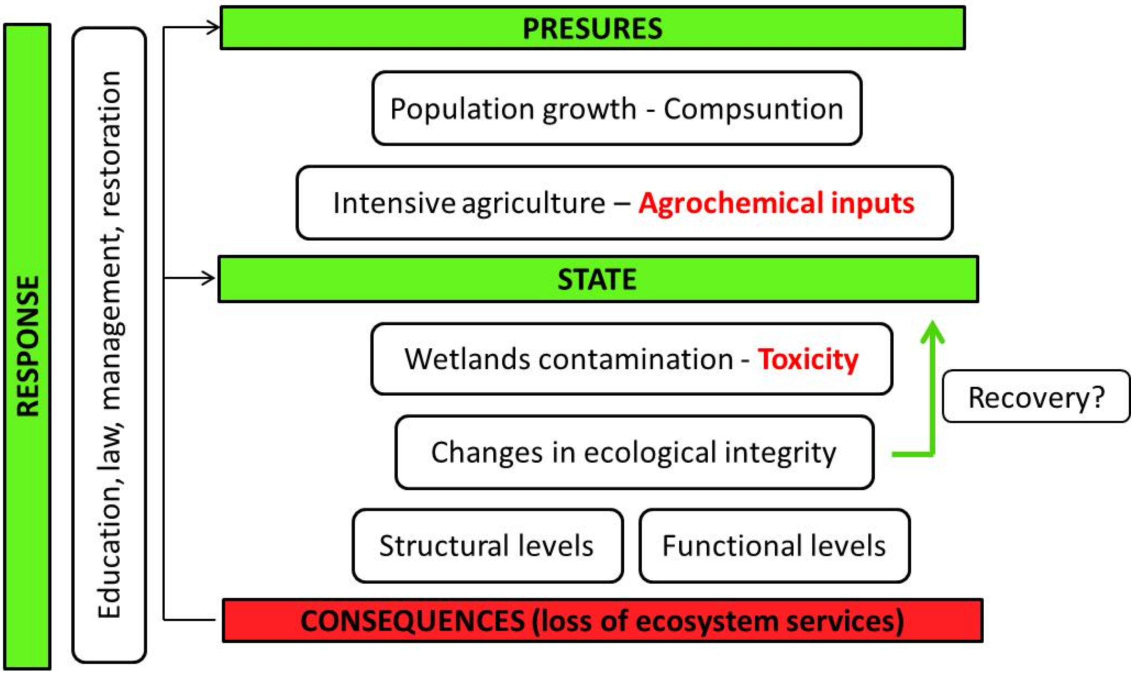

Figura 2. Modelo conceptual del marco en que se encuadra la tesis.

Por tanto, la tesis se centra en una de las presiones de la agricultura intensiva sobre las masas de agua: la contaminación por agroquímicos. Debido a que los agroquímicos general cambios en el estado del medio ambiente a través de su toxicidad, el estudio se consideró dentro de la ecotoxicologia. La ecotoxicologia es “una subdisciplina de la ecología que se centra en los efectos de sustancias tóxicas en los ecosistemas y en sus componentes vivos” (Jorgensen, 2010). Los estudios ecotoxicológicos están ayudando a entender las respuestas de los individuos, poblaciones y comunidades a las presiones químicas, proporcionando evidencias científicas útiles para la toma de decisiones. En este sentido, la UE hace esfuerzos para caminar hacia un buen estado químico y ecológico de todas las masas de agua en 2015 (WFD 2000/60/CE). Una de las herramientas para evaluar los impactos químicos en los ecosistemas es la Evaluación del Riesgos Ecológico de los productos químicos (en inglés Ecological Risk Assessment ERA).

Los ERA son un conjunto de diferentes técnicas y metodologías (Figura 3) que permiten examinar y evaluar los efectos en los ecosistemas y los seres vivos, incluidos los humanos, la exposición a tóxicos (EEA, 2014). La ecotoxicologia está ligada y debe dirigirse principalmente a la evaluación del riesgo ecológico. Frecuentemente, la caracterización y evaluación de los efectos se basa en el cálculo de la PNEC (Concentración prevista sin efecto, siglas en ingles de Predicted-No Effect Concentration) que indica el nivel de exposición al que no se producen efectos adversos sobre el ecosistema. La manera más fácil de calcular el PNEC es usar el método del cociente (en inglés, Risk Quotient) comparando la toxicidad con la exposición ambiental. En este método, la estimación de la concentración ambiental (EEC) se compara con un nivel de efecto, como el LC50 (la concentración de tóxico a la que muere el 50% de los organismos expuestos). Cuando el cociente es mayor a 1, existe riesgo de toxicidad. Inicialmente, los límites de agroquímicos se han basado en test de toxicidad de laboratorio que evaluaban los efectos de la exposición a un tóxico basándose en criterios de valoración (supervivencia, crecimiento y reproducción) de una sola especie expuesta sólo a un compuesto, como la clásica LC50. Pero, hay dos limitaciones principales asociadas con este enfoque: a) la falta de realismo de la extrapolación de los efectos basados en criterios de valoración a nivel individuoal hacia niveles de mayor complejidad en el ecosistema como las poblaciones o las comunidades (Van den Brink, 2013; Wootton, 2002; Brooks *et al.*, 2009), y b) que las sustancias químicas no aparecen individualmente en el medio, consecuentemente las mezclas deberían ser objeto de estudio para entender mejor tanto efectos directos como indirectos de los agroquímicos (LeBlanc *et al.*, 2012; Anderson *et al.*, 2006; Barata, 2006).

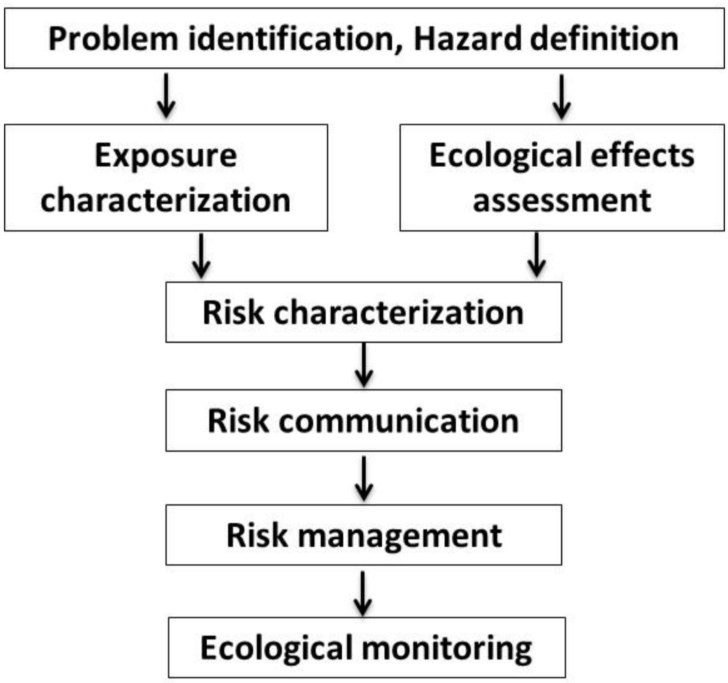

Figura 3. Diagrama conceptual de la evaluación de riesgos ecológicos (ERA) junto con los riesgos en comunicación, gestión y monitoreo ecológico (Jorgensen, 2010).

Organizaciones como la Organización para la Cooperación y Desarrollo Económicos (OECD), la Organización Mundial de la Salud (OMS) y el Centro Europeo de Ecotoxicología y Toxicología de Productos Químicos (el acrónimo se usa en inglés, ECETOC) trabajan de manera activa en la mejora de ERA para facilitar la toma de decisiones en el ámbito de la gestión (EEA, 2014). De hecho, se sabe de manera informal que las directivas con objetivos de protección de la UE basadas en resultados a nivel de individuos, necesitan aumentar su nivel de complejidad para alcanzar dichos objetivos de protección con los niveles de población y comunidad en los ecosistemas (De Laender *et al.*, 2013). De Laender *et al.* (2013) destaca 5 áreas primordiales de estudio para equilibrar la asimetría mencionada entre la información utilizada y los objetivos de protección: estudiar la influencia de 1) la competencia y 2) la depredación en poblaciones y comunidades así como en la capacidad de recuperación; 3) explorar los efectos de los químicos en la biodiversidad; 4) determinar los efectos en los servicios de los ecosistemas y sus funciones tras exposción a químicos; y 5) evaluar los efectos de las mezclas de tóxicos. La evaluación del riesgo ecológico pretende establecer estándares en el uso de productos químicos para prevenir el impacto ambiental. Para dicho fin, los procedimientos seguidos en la evaluación del riesgo ecológico debería necesitar una actualización considerando las 5 principales áreas de estudio mencionadas arriba para establecer límites legales capaces de proteger lo suficiente al ecosistema. Con respecto a esto, los límites legales actuales están basados en test realizados con una sola especie por lo que carecen de realismos ecológico y químico. Por lo tanto, no serán adecuados para obtener evaluaciones de riesgo ecológico realistas con los que evaluar efectos tanto directos como indirectos (Crane, 1997; Boxall *et al.*, 2002). La existencia de estas debilidades en los sistemas actuales de evaluación se presentan como un complejo reto en el que se debe aceptar varias limitaciones: a) es extremadamente complicado asumir el coste de todos los impactos ambientales y, b) los gestores de medio ambiente tienen que tomar decisiones asumiendo un alto nivel del incertidumbre (Jorgensen, 2010). La actitud para afrontar esta alta complejidad no debe ser la desmotivación de llevar a cabo acciones para prevenir impactos medio ambientales. Por el contrario, la respuesta ante dicha complejidad debe ser invertir en investigación para tomar decisiones con una mayor base científica y, sobre todo, poner en práctica el principio de precaución cuando no tenemos suficiente información.

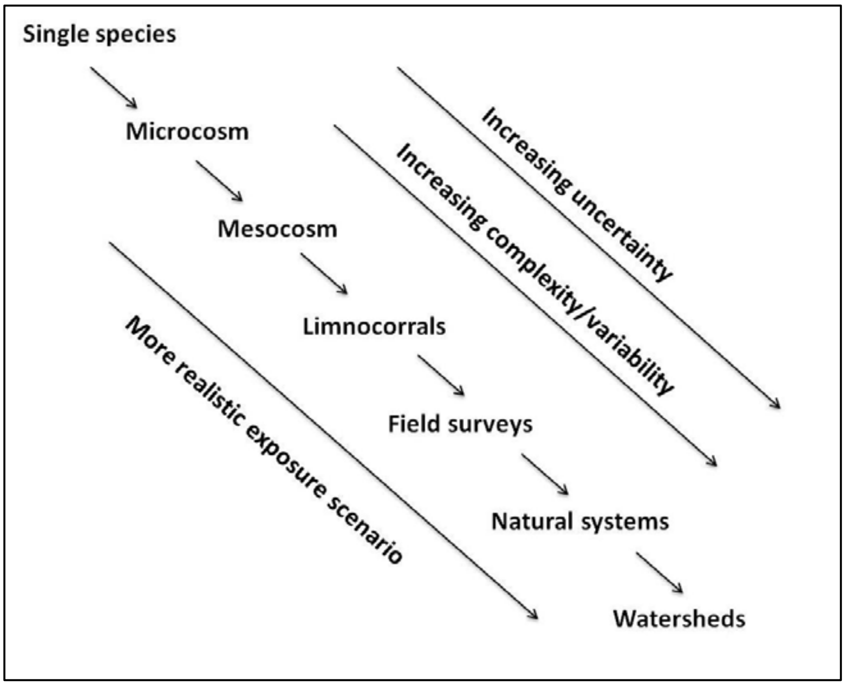

Figura 4. Diferentes test ecotoxicológicos usados para establecer concentraciones biológicamente seguras de tóxicos en medios acuáticos (Modificado por Jorgensen, 2010). El diagrama muestra el aumento de la incertidumbre y la complejidad cuando se realizan estudios más realistas desde el punto de vista ecotoxicológico.

Escenarios de la tesis

Esta tesis explora escenarios de mezcla de químicos, aspectos ecológicos y sus interacciones y como la primera regla para gestionar escenarios complejos es simplificar, se plantearon distintos contextos. Con el objetivo de contribuir a una mejor comprensión de los efectos de los agroquímicos en las comunidades acuáticas, este trabajo aúna dos escenarios principales a través de 6 capítulos: escenarios de agroquímicos (mezclas, frecuencia de pulsos y límites) y escenarios ecológicos (interacciones ecológicas: competición y depredación, y niveles jerárquicos) (Figura 5).

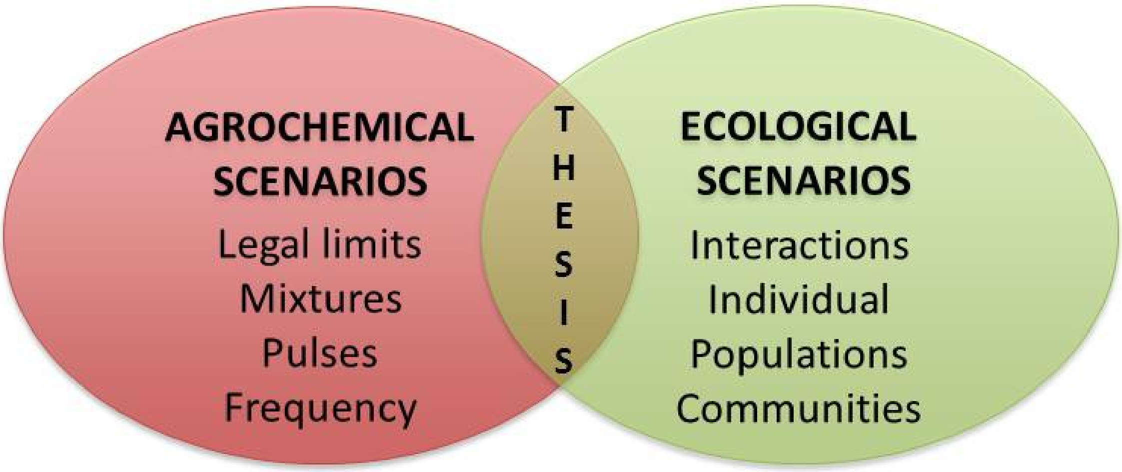

Figura 5. Escenarios principales contemplados en la tesis.

El primer objetivo trata la complejidad de escenarios de exposición de agroquímicos en el medioambiente que dependen principalmente de los usos agrícolas, factores ambientales (topografía, climatología, etc) y los límites legales. La concentraciones de químicos en eventos de escorrentía varían en función de la estación y de los tiempos de aplicación. Las interacciones entre tóxicos es un escenario más frecuente de que haya un único tóxico, por tanto debería ser estudiado (Kungolos *et al.*, 2009). Tal interacción de efectos podría ser: aditiva cuando los químicos no interaccionan y el efecto es la suma de los efectos de su toxicidad individual; sinérgica cuando hay interacción entre químicos y la combinación de sus efectos es mayor que si ocurriese una interacción aditiva; y, antagónica cuando los químicos interaccionan y el efecto tóxico de uno de ellos se contrapone al efecto tóxico del otro. Por otra parte es muy importante explorar los efectos de las bajas concentraciones de químicos que se detectan de manera rutinaria en el medio ambiente (Le Blanc *et al.*, 2012) los cuales pueden estar dentro de los límites legales pero aún así afectar negativamente a los sistemas acuáticos. Esta última necesidad de investigación está actualmente en auge y va más allá del objetivo de esta tesis. Ya que lo mencionado plantea la pregunta de si niveles de contaminación subletales detectados rutinariamente podrían influenciar la microevolución o adaptaciones locales hacia poblaciones y/o especies más tolerantes.

Los agroquímicos usados en agricultura alcanzarán los ecosistemas acuáticos tanto directamente por pulverización como por eventos de escorrentía (Brock *et al.*, 2006).

El modo de acción (pesticidas, fungicidas, fertilizantes…) y las características químicas (secuestro, detoxificación, bioacumulación, sinergia…) tienen repercusión en los efectos finales del tóxico y por tanto en la evaluación del riesgo ecológico. Una sección de los experimentos presentados considera la modulación de toxicidad, considerando concentraciones de los tóxicos por encima y por debajo de los límites legales, con el fin de determinar el posible riesgo ambiental. El uso de concentraciones por encima de los límites legales se justifica porque las concentraciones de los contaminantes puede aumenta varios órdenes de magnitud tras periodos de lluvias (Rabiet *et al.*, 2010). El interés de estudiar concentraciones de tóxicos por debajo de los límites legales proviene de la posibilidad de que ocurran efectos subletales debido a dosis bajas rutinariamente detectadas en el medio ambiente (Maltby *et al.*, 2002; Le Blanc *et al.*, 2012). Por ejemplo, una consecuencia de efectos subletales podría ser el desarrollo de tolerancia de específicos estadios de vida o de determinadas especies, lo que vendría a significar una desviación de lo que ocurriría en zonas no contaminadas. Brausch y Salice (2011) publicaron que la segunda generación de *Daphnia magna* no se veía afectada por bajas concentraciones de pesticidas medidas en el medio (realistas), lo que sugiere el desarrollo de tolerancia. Por tanto, concentraciones de tóxicos por debajo de límites legales podrían tener efectos subletales que pueden ser un punto crítico para entender mejor los efectos en campo a nivel de población y de comunidad. A parte de las concentraciones de tóxicos, los pulsos de tóxicos son también un factor relevante cada vez más importante en los estudios de ecotoxicología. La mayoría de los estudios evalúan concentraciones de tóxicos constantes (Hecnar 1995; García-Muñoz *et al.*, 2009), sin embargo, la aplicación de pequeños pulsos sumando la misma concentración final, podría tener efectos diferentes (Earl and Whiteman, 2009; García-Muñoz *et al.*, 2011). En condiciones de campo, la exposición de los organismos acuáticos a la contaminación fluctúa en concentración, duración y frecuencia (Hoang *et al.*, 2007; Downing *et al.*, 2008). La entrada de agroquímicos en sistemas acuáticos varia principalmente debido a: escorrentía relacionada con periodos de lluvia; y, el tiempo de aplicación dependiendo en los requisitos de crecimiento del cultivo y del control de plagas y enfermedades (FAO 2006; Le Blanc *et al.* 2012; Haygarth *et al.* 2012; Reinert *et al.* 2002; Hoang *et al.* 2007; Earl and Witheman, 2009). Por dicha razón, los experimentos presentados en esta tesis exploran tanto exposiciones de un único pulso como de varios.

En otra sección de la tesis se plantean experimentos que tratan sobre la respuesta obtenida tanto a un único químico como a la mezcla de varios de ellos. Por ejemplo, en el Delta del Ebro se detecta de manera rutinaria más de 30 pesticidas en aguas superficiales (Suárez-Serrano *et al.*, 2010), sin embargo, los efectos de los pesticidas en organismos acuáticos se basan en ensayos con un único tóxico (Lydy *et al.*, 2004). Es obvia la alta complejidad de extrapolar los resultados de Evaluaciones de riesgo ecológico (ERA) a condiciones de campo considerando resultados de test que usan un único tóxico. Por tanto, a pesar de que considerar mezclas sería más apropiado, la complejidad aumentaría exponencialmente. No obstante, los estudios de mezclas son necesarios para paliar el desconocimiento ante escenarios de exposición complejos. De hecho, la Comisión Europea subraya que solo unos pocos estudios, clasificados como de semi-campo, evalúan los efectos de la mezcla de pesticidas en organismos acuáticos y en las funciones de los ecosistemas (European Commission, 2006). Esta tesis explora los efectos tóxicos de fertilizantes (nitrato amónico, nitrógeno y fosforo), fungicidas (sulfato de cobre y carbendazim) e insecticidas (clorpirifós). La selección de los agroquímicos responde a diferentes criterios. El nitrato amónico y el sulfato de cobre se eligieron debido a su relevancia local en el cultivo del olivo en Jaén (Andalucía) así como por su uso global en otros tipos de cultivos. A pesar de que los nitratos aparecen de manera natural en sistemas acuáticos, sus concentraciones han crecido debido al uso de fertilizantes en la agricultura intensiva. García-Muñoz *et al.* (2011) encontaron efectos en la supervivencia y la longitud total en anfibios (*Epidalea calamita*) debido a la exposición de pulsos subletales de nitrato. En el caso del sulfato de cobre, se puede aplicar incluso directamente sobre sistemas acuáticos como herbicida o alguicida a nivel mundial. Además, el cobre, al ser un metal pesado, pertenece a una categoría superior de contaminantes que tienen impactos también sobre la salud humana (Duruibe *et al.*, 2007). El sulfato de cobre es el producto comercial que se usa en agricultura, pero el cobre es el químico en el que se centran los experimentos. Aunque el cobre se puede encontrar de manera natural en diferentes formas, éste puede ser tóxico en sistemas acuáticos como Cu^{2+} (Lenwood *et al.*, 1998). Estudios previos han puesto de manifiesto efectos directos en individuos y a largo plazo en poblaciones. Parra *et al.*, (2005) muestran los efectos del cobre en las tasas de eclosión y la supervivencia de los nauplios del copépodo *Arctodiaptomus salinus*; mientras que, Johnston and Keough (2005) encuentra cambios en la estructura de la población de invertebrados sésiles marino como resultado a la exposición de pulsos de cobre.

Los nutrientes (nitrógeno y fósforo) y el clorpirifós son los agroquímicos seleccionados para estudiar los escenarios de mezclas, pulsos y la frecuencia de pulsos. El ciclo del nitrógeno ha sido profundamente alterado por las actividades humanas conllevando numerosas entradas de nitrógeno en sistemas acuáticos a través de la escorrentía desde los campos de cultivo, la producción de ganado o la deposición atmosférica (Gallaway *et al.*, 2004). El ciclo del fósforo ha sido también alterado por la actividad humana suponiendo un aumento de su liberación desde los sedimentos (Mooij *et al.*, 2005) o entrando en los sistemas acuáticos a través de escorrentía durante la estación de fertilización de los cultivos, y consecuentemente alterando los sistemas acuáticos naturales. Por ejemplo, Miracle *et al.* (2007) propuso una relación entre entrada de nutrientes en sistemas acuáticos y un cambio hacia fases de aguas turbias como resultado de los cambios en la comunidad zooplanctónica inducidos por los cambios en la concentración de nutrientes. Estos cambios en las concentraciones de nutrientes tienen lugar junto a otras presiones químicas como la contaminación por insecticidas. Clorpirifós es un insecticida organofosforado de amplio espectro usado en agricultura a nivel mundial. Brock *et al.* (2000) publicaron efectos tóxicos del insecticida en el crecimiento, la supervivencia y la reproducción de organismos acuáticos. Actualmente existe una alta probabilidad de confluencia de Clorpirifós y distintos nutrientes en sistemas acuáticos rodeados de agricultura, debido a sus similares tiempos de aplicación acabando juntos en el medio acuático gracias a los eventos de escorrentía (FAO 2001; FAO 2006; Reinert *et al.*, 2002). Por otra parte, Carbendazim es un fungicida usado mundialmente en el control de plagas en colza oleaginosa, maíz y arroz entre otros cultivos. Se ha descrito previamente que el carbendazim tiene efectos negativos en especies de macroinvertebrados (Cuppen *et al.*, 2000) y especies de plancton (Van den Brink *et al.*, 2000). Los diferentes tipos de agroquímicos mencionados han mostrado efectos negativos sobre diferentes organismos lo que podrían afectar a niveles jerárquicos superiores mediante, por ejemplo, cambios en la estructura de la comunidad. Se han publicado varios estudios que abarcan la complejidad de las comunidades como los de Van den Brink *et al.* (2000) y Downing *et al.* (2008). Van den Brink *et al.* (2000) evaluó los efectos de un fungicida (carbendazim) en el zooplancton y en productores primarios. Estos investigadores encontraron cambios estructurales como consecuencia tanto de efectos adversos directos del tóxico en organismos zooplanctónicos y en macroinvertebrados, como de efectos indirectos en el crecimiento del fitoplancton debido a una disminución en la presión de herbivoría. También Downing *et al.* (2008) estudiaron la respuesta de una comunidad de aguas dulces a pulsos realistas (medidos en el medio) de un pesticida (Sevin) y encontraron una disminución de los indicadores de zooplancton (riqueza, diversidad, abundancia y concentración de oxígeno) mientras que hubo un aumento de los indicadores de abundancia del fitoplancton y de la actividad microbiana.

El segundo escenario se centra en aspectos ecológicos. La evaluación del riesgo ecológico se basa en test con especies estándar cuyos resultados se usan para establecer límites legales para sustancias químicas. Sin embargo, una limitación principal de este tipo de test es la falta de relevancia ecológica al ignorar las interacciones ecológicas y al usar sólo especies estándar en lugar de especies locales. Por tanto, la mencionada debilidad compromete la extrapolación de resultados a nivel jeráquicos superiores. En este sentido hay que recalcar que el objeto de estudio de esta tesis es la comunidad acuática. Es sabido que los pesticidas y los fertilizantes pueden impactar la integridad de la comunidad acuática. Los cambios en su estructura y funciones se pueden usar como indicadores para evaluar los efectos tóxicos a esos niveles jerárquicos. Tradicionalmente, se ha informado sobre efectos negativos a niveles morfológicos, fisiológicos, bioquímicos o genéticos (Troncoso *et al.*, 2000). Esto consecuentemente, puede alterar la reclusión de especies, las tasas de eclosión, las tasas de supervivencia así como la capacidad de herbivoría (Parra *et al.*, 2005; Sharp y Stearns, 1997), lo que agudiza la preocupación sobre las consecuencias a largo plazo para los ecosistemas y los servicios que estos proveen. En este sentido, algunos experimentos de campo han relacionado eventos de alta proliferación de algas con la disminución de invertebrados herbívoros afectados por insecticidas (Hurlbet *et al.*, 1972, Boyle *et al.*, 1996). Dicha proliferación de algas puede desembocar en problemas de eutrofización alterando la calidad del agua, la vegetación sumergida y la biodiversidad (Miracle *et al.*, 2007) consecuentemente, disminuyendo los servicios de los ecosistemas. Los efectos de agroquímicos en los sistemas acuáticos se han regulado con un enfoque basado en especies estándar (por ejemplo, *Daphnia* sp. or *Chironomus* sp. para invertebrados, algas verdes para algas or *Lemna sp*. para macrófitos). No obstante, se puede defender con un alta probabilidad que los agroquímicos afectaran las comunidades locales de manera distinta al limitado grupo de especies estándar más estudiado. Con el objetivo de superar esta limitación, se usaron experimentos de microcosmos con ensamblajes de comunidades locales. Los microcosmos permitieron establecer ensamblajes biológicos naturales para evaluar la exposición de los tóxicos a niveles de población y comunidad (Figura 4). Los estudios con múltiples especies no son abundantes a pesar de que potencialmente dan datos más relevantes ecológicamente comparados con los obtenidos con test de una única especie. Los microcosmos presenta la posibilidad de además, evaluar efectos indirectos, capacidad de recuperación y relaciones estructurales-funcionales (OECD, 2006; Sanderson *et al.*, 2009). Por tanto, los microcosmos establecidos con poblaciones que coexisten en humedales naturales locales se consideraron una metodología adecuado para los objetivos de la tesis. Asimismo, microcosmos con un nivel de complejidad más bajo también se han usado para explorar relaciones ecológicas específicas como la competencia. El uso de ciertos estudios sencillos sobre interacciones ecológicas en combinación con experimentos a nivel de comunidad puede ayudar a identificar mecanismos que controlen tanto respuestas directas como indirectas a la exposición de agroquímicos. Esta combinación de estudios experimentales podría permitir una interpretación holística de los efectos a niveles jerárquicos de mayor complejidad. En este contexto, es crucial considerar factores biológicos y ecológicos como lo son las especies presentes, el genotipo, el estadio de vida y las relaciones ecológicas (Hanazato *et al.* 2001; De Laender, 2013).

Las interacciones ecológicas son un área de investigación que recientemente se ha recomendado incluir en las evaluaciones de riesgo ecológico (Van den Brink, 2013; De Laender *et al.*, 2013; Brocks *et al.*, 2006). Así se contribuirá a no subestimar o sobreestimar el riesgo que suponen los agroquímicos sobre las comunidades acuáticas (Pestana *et al.*, 2009; Foit *et al.*, 2012).

A pesar del reconocimiento emergente del papel que cumple los ecosistemas acuáticos de menor tamaño (tal como, lagos y charcas pequeñas como es el caso de la mayoría de los humedales de Jaén) en procesos y ciclos globales, la investigación sobre este tipo de sistemas relativa a ecotoxicología es un área aún inexplorada (Downing *et al.*, 2010). Dicho desconocimiento es la justificación para que el presente estudio se centre en ellos. En estos sistemas acuáticos, el plancton (fitoplancton y zooplancton) es un componente importante porque es una vía principal en el flujo de energía (Alvarez-Cobelas and

Rojo, 2000; Nayar *et al.*, 2004). El fitoplancton constituye el productor primario, así pues, su estructura y su dinámica influencian a niveles tróficos superiores como es el zooplancton. Los cambios en la comunidad de fitoplancton podrían dar lugar aumentar los taxones incomestibles para la comunidad del zooplancton. Una disminución de la capacidad de herbivoría del zooplancton es de extrema importancia porque puede desencadenar en procesos de eutrofización (Van Wijngaarden *et al.*, 2005; Hanazato, 1999; Fleeger *et al.*, 2003). El zooplancton representa uno de los mayores componentes en sistemas acuáticos y tiene una influencia tanto en la calidad del agua como en otros niveles tróficos. En estudios previos se ha subrayado las consecuencias de los cambios en la abundancia y en la estructura comunidad sobre la fase de aguas claras especialmente durante la primavera de los lagos (Hanazato, 1998) y su repercusión en el desarrollo de las larvas de peces (Zagarese, 1991). En esta tesis también se han usado organismos bentónicos, el criterio de selección de estos taxones se basa en su importancia ecológica así como en otros aspectos relevantes para el diseño experimental como es el que coexistieran en la naturaleza y la fácil manipulación de los organismos en laboratorio. Por tanto, los experimentos de competencia se llevaron a cabo usando gasterópodos (*Bithynia tentaculata* y *Radix peregra*), anfípodos (*Gammarus pulex*) e isópodos (*Asellus aquatic*). En el caso de los gasterópodos, su relevancia ecológica se debe a su alta abundancia en sistemas acuáticos, que supone entre un 20%-60% de la biomasa total de invertebrados en algunos ecosistemas de aguas dulces (Habdija *et al.*, 1995). Por otro lado, los anfípodos y los isópodos se consideran dos de los mayores descomponedores de hojarasca, lo que es crucial para evaluar aspectos funcionales de los ecosistemas (Zubrod *et al.*, 2010; Graça *et al.*, 1994).

Con las secciones y párrafos anteriores defendemos la necesidad de esta investigación, el marco conceptual, los escenarios, la metodología, así como la selección de agroquímicos y especies utilizadas. Lo que permite a continuación el planteamiento de la hipótesis de partida y los objetivos.

HYPOTHESIS AND OBJECTIVES

The working hypothesis is that agrochemicals exposure due to prevailing agriculture intensive practices **has negative effects** on the aquatic community integrity at both structural and functional levels.

The main objectives are:

- **OBJECTIVE 1.** Assess the effect of agrochemicals commonly used **above and below legal** limits on aquatic community in order to test if current legislation over- or under protect aquatic communities.
- **OBJECTIVE 2.** Assess how agrochemicals **mixture and pulses frequency** effects on aquatic community vary compare to single agrochemical exposures.
- **OBJECTIVE 3.** Assess the influence of **ecological interactions** on the sensitivity response of aquatic species to agrochemicals.

These objectives have been considered through the six chapters of this thesis. Chapters 1, 2, 3 and 4, deal with single and mixture exposure of ammonium nitrate and copper sulfate and have the plankton community as studied subject, considering the effects on its structure and function using microcosms. Chapter 5 has the plankton community as studied subject; changes in its structure and function were assessed using microcosms but chlorpyrifos and nutrients were used as toxicants. However, all of them are related with objectives 1, 2 and 3, and embraces both agrochemical and ecological scenarios. Finally, chapter 6 presents two competition experiments with just one toxic substance, deepening mainly in aspects related to ecological interactions so objective 3. Each chapter corresponds to articles that have been published, sent or are submitted to scientific journals. In order to facilitate the link between the objectives and the chapters the articles are cited below:

Chapter I: Shifts across trophic levels as early warning signals of copper sulfate impacts in plankton communities.

Chapter II: Could a single copper sulfate pulse within legal limits change plankton community's features?

Chapter III: Effects of nitrate concentrations within legal limits on natural assemblages of plankton communities.

Chapter IV: Effects of environmental relevant agrochemical mixtures within legal limits on planktonic community.

Chapter V: Zooplankton community response and recovery to disturbance variability: the importance of pulses, frequency and synchrony of agrochemical mixtures in wetlands.

Chapter VI: Effects of intra- and interspecific competition on the sensitivity of aquatic macro-invertebrates to carbendazim.

Understanding agrochemical effects on aquatic systems provides knowledge to improve Ecological Risk Assessment. It could also enhance policy makers to legislate based on science-evidences. These two statements linking basic and applied science were the engine of the thesis and its results and conclusions are presented in the following chapters.

CHAPTER 1

"Shifts across trophic levels as early warning signals of copper sulfate impacts in plankton communities"

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SHIFTS ACROSS TROPHIC LEVELS AS EARLY WARNING SIGNALS OF COPPER SULFATE IMPACTS IN PLANKTON COMMUNITIES

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Abstract. Intensive agricultural practices have been characterized by an overuse of agrochemicals. The inputs of chemicals in a watershed are likely to alter trophic interactions affecting its ecological integrity. This ecotoxicological study aims to identify warning signals of agrochemicals effects on a plankton community. Eighteen outdoor microcosms were used to establish an experiment with 2 concentrations of copper sulfate above and below the legal limit with six replicates lasting two weeks. Phytoplankton and Zooplankton structure changes were studied. Chlorophyll-a concentration was used as a functional indicator. A rapid change in phytoplankton structural features, abundance and size classes, was detected after both, below and above legal, concentrations. Similarly, Zooplankton structural changes showed an effect of copper exposures on abundance and composition. As Phytoplankton response was so rapid, it could be used as an early and direct warning signal, but also to warn of future indirect effects on zooplankton structural features owing to a change of food resources. In summary, the shifts across both trophic levels could be use as warning signals. Moreover, even legal limits do not protect the plankton community, which emphasises the need of a more ecotoxicological and realistic approach to achieve a balance between agriculture development and ecosystems conservation.
Key words: *Copper; Microcosms; Plankton; Warning*

Introduction

Current main environmental pressures are highly linked to human exponential population growth (United Nations Population Division 2000). One of the main consequences of population growth is an increase in food demand; therefore, agricultural practices intensify with the consequent increased use of agrochemicals (Valavanidis & Vlachogianni 2010). The socio-economic value of agriculture cannot be denied, however, its integration with environmental criteria must be a priority. The improper use and / or application in excess of pesticides, herbicides and fertilizers generate an impact on the ecological integrity of ecosystems, affecting their structure and function (Troncoso et al. 2002, Parra et al. 2005). In this sense, there is enough bibliographic information which shows that intensive agriculture is causing the disappearance and / or pollution of wetlands (Casado & Montes 1995, Troncoso et al. 2000, Parra et al, 2005, García-Muñoz et al. 2011). These impacts have consequences at different hierarchical levels: from the individual by morphological, physiological and biochemical alterations, to the community level through the loss of diversity, and impairing the value and services that healthy ecosystems provide (Montes & Sala 2007).

Ecological indicators are tools which detect changes on ecosystems that are likely to impact ecological integrity and so then the community structure and function, and consequently ecosystem services. Structural features have been usually used alone to monitor and assess impacts on the ecosystem ecological integrity, focusing on communities assemblages and their resources. Structural attributes are easiest to visualize and they are, by far, the most commonly used. Examples are species numbers, dominant species, guild composition, taxonomic representation, abundances, size composition, and others (Bain et al. 2000). For instance, crustacean zooplankton size has been shown to be more strongly correlated with lake water quality than community taxonomic structure (Sprules 1984). In addition to structural levels, ecosystem functional aspects are gaining more importance in the evaluation of ecosystem integrity. Functional indicators assess rates and patterns of ecosystem processes and are considered to be an essential complementary aspect to assess ecological integrity due to their different sensibility from structural levels to environmental pressures (Gessner & Chauvet 2002). However nowadays, society is demanding this to go further, with the intent of detecting, as soon as possible, the negative effects on ecosystems. In this sense, a warning signal is an important component of the integrated approaches that are needed to acquire a general knowledge of toxic impact, and which will allow predictions and early mitigation measures (Schmitt-Jansen et al. 2008).

The study is focused on the effects of a fungicide (copper sulfate) on trophic levels in wetlands which are surrounded by intensive olive tree agriculture. The aim was to evaluate how this toxic substance could alter structural and functional characteristics of the plankton components, and if these changes could be used as early warning signals. In the present study two different concentrations were used, the first above the legal limit, in order to find clear effects on the plankton community features. The second concentration, below the legal limit, in order to check if the changes could be detected even before the community was highly altered.

Materials and methods

Microcosms

Eighteen microcosms were set, based on, and adapted from, OECD (2006), and were placed outdoors in a specific installation at the University of Jaén (HUMEXPUJA, experimental wetland infrastructure in the University of Jaén, which were exposed to the same environmental conditions). Microcosms length, height and width were 0.34-0.28-0.24 cm respectively, 22.8 liters in volume and placed 15 cm apart from each other. Microcosms were filled with 18 liters of water and 5 cm of sediment. Water came from an artificial pond supply free of contamination and zooplankton (HUMEXPUJA). Sediment came from a natural wetland [Casillas wetland, UTM 30SVG1084 with a surface area of 2.2 ha. (Ortega et al. 2003)], it was homogenized and distributed among the microcosms. Microcosms were established in November 2011 and the experiment was finished in January 2012. There was a stabilization period of 7 weeks before adding copper to the microcosms in order to favour the development of the planktonic communities from the resistant structures present in the sediment. The experiment lasted 21 days, with a single pesticide spike on day 0.

Disturbance

Control and two treatments of copper sulfate, with six replicates each one, were used in the experimental design. The first one, called high treatment (H: 0.2 mg l^{-1} Cu), represents a concentration of copper sulphate over the limit established by both the Water Framework Directive (WFD 2000/60/CE) and its application into the Spanish National legislation (DMC 2000/60/CE) (0.04 mg l^{-1}). The second one, low treatment (L: 0.02 mg l^{-1} Cu) shows a lower concentration than those legal limits previously mentioned. Therefore, our L treatment falls within legal limits, while the H treatment is one order of magnitude higher. Nominal dosages of copper sulfate were directly added and stirred over the water surface of the microcosms as an only pulse on day 0 for the whole experimental period. The criteria to establish the concentration of the treatments was not based on lethal concentration data of the species involved because the aim of the study focused on studying the effect over the entire plankton community. Water samples to control the fate of copper sulphate were taken every week and analyzed by ICP Mass Spectrometry.

Physical-chemical variables

Each microcosm was surveyed every seven days. Each time, physical-chemical measurements (temperature, pH, % dissolved oxygen and conductivity) were taken using field probes. At the same time, water samples were taken, cold stored and transported to the laboratory to perform nitrogen dissolved nutrients (nitrate) and alkalinity analysis. Alkalinity was measured in the lab using a 848 Tritino Plus device. Nitrate was determined following the reduced column Cadmium method (Keeney & Nelson 1982).

Biological variables

Abundance and changes in phytoplankton size distribution were evaluated with flow cytometry. Water samples were taken weekly, preserved in glutaraldehide (4%), frozen in liquid nitrogen and stored at 80ºC until running the analysis with BD- LSR Fortessa flow cytometer. Calibration spheres were used to obtain a cell size regression curve: $y = 0.011\ x - 14{,}388$, where "x" represents the mean of the Forward Scatter (FSC), and "y" represents the cell size of the cells in μm^3. Three cell size populations were determined characterized by a mean volume of 58 μm^3 (small), 304 μm^3 (medium) and 749 μm^3 (high). Population cells abundance were determined from an acquisition time of 180 s at a rate of 60 $\mu L\ min^{-1}$. Data analysis was performed using the FACSDIVA software.

Chorophyll-*a* concentration was measured weekly with a field fluorometer (Aquafluor deTurner Design). Chlorophyll-a (Chl-a) concentrations were later calculated using a previously obtained calibration curve determinate by fluorometry. Calibration samples were filtered through Whatman GF/C glass microfibre filters (1.2 µm pore-size), and extracted in 90% acetone for 24 h at 4ºC (Strickland & Parsons 1968).

Zooplankton in microcosms was sampled weekly during the study through water-integrated samples of 100 ml. Water integrated samples were collected, then filtered through a plankton net of 30 µm, and preserved *in situ* with formalin (4%). The filtered water was returned to the microcosm. Zooplankton was identified to the lowest practical levels and abundance estimated.

Physical-chemical, plankton and Chl-*a* variables were compared among microcosms using univariate and multivariate analyses with SPSS 19 software. Repeated measures of ANOVA were used to test for time and time x treatment effects. An univariate ANOVA and a post hoc Tukey test at the sampling date were used to determine the significance of differences between treatments. Prior to analysis, data were tested for normality and homoscedasticity. Zooplankton data could not be treated with a parametric test due to its low abundance or even complete disappearance in some microcosms. Therefore, total zooplankton abundance, rotifera abundance, and copepod abundance were analyzed with the non-parametric test of Friedman to test for differences due to time and treatment. Wilcoxon post hoc test was also used to determinate which treatments were significantly different from one another. Ordination of treatment and control of physical-chemical parameters and biological variables, except phytoplankton cell size populations, were made considering a Principal Component Analysis (PCA) (CANOCO v4.5 software). PCA aimed understanding the main factors influencing microcosm's responses.

Results

Copper nominal concentrations were achieved with the spike on day 0. The degradation was very low, therefore the average concentration exposure over the whole experiment matched the intended nominal concentrations (*Table 1*).

***Table 1**. Mean ± standard deviation (S.D.) of copper sulfate after pulse, by the end of the experiment and the average concentration exposure.*

Nominal concentration (mg L^{-1})	Concentration (mg/l) after pulse application (day 0) ± S.D.	Concentration ((mg/l) after pulse (day 14) ± S.D.	Average concentration exposure (mg/l) ± S.D.
0	0.01± 0	0.01 ± 0	0.01 ± 0
0.02	0.03 ± 0.01	0.09 ± 0.04	0.06 ± 0.05
0.20	0.11 ± 0.09	0.18 ± 0.04	0.14 ± 0.07

Temperature ranged from 9ºC to 13ºC during the experimental period. Dissolved oxygen (% DO), pH, conductivity and alkalinity presented significant differences between treatments and controls, while nitrate concentration did not present significant differences among them, independently of the treatment (*Table 2*). At the same time, pH was higher in H treatments in day 0 ($F = 396.820$, $P = 0.000$) and in L and H treatments in day 7 ($F = 236.197$, $P = 0.000$). Dissolved Oxygen (%) was lower in L and H treatments from day 0 ($F = 148.684$, $P = 0.000$) till the end of the experiment in day7 ($F = 143.703$, $P = 0.000$). Average oxygen content in controls, L and H treatments were 16, 13 and 12 mg l^{-1} respectively. Conductivity was higher in L and H treatments from day 0 ($F = 4.104$, $P = 0.038$) till the end of the experiment in day7 ($F = 6.273$, $P = 0.010$). Alkalinity was higher in L and H treatments in day 7 ($F = 43.707$, $P = 0.000$). PCA shows that those differences were not relevant enough to discriminate among treatments. PCA of physical-chemical and biological variables discriminate the controls (to the left) from the treatments (to the right) (*Fig. 1*). The two main axes explain 89% of the variance, x-axis explains 69% and y-axis explains 20% and they are correlated to conductivity and copper concentration and to rotifera and zooplankton abundance, respectively.

The results obtained show a negative effect of both copper sulphate concentrations tested on the plankton community under study. Even legal limits do not protect the plankton community. The plankton community was affected by a decrease in phytoplankton and zooplankton abundances under both copper concentrations.

***Table 2**. Physical-chemical and biological parameter measurements (mean ± SE) in treatments and controls microcosms along the whole experiment period. *Denotes statistical significant differences with the controls.*

Treatments	Controls			Low			High		
Parameters / Days	0	7	14	0	7	14	0	7	14
Temperature	14.23 ± 0.59	7.26 ± 0.08	10.44 ± 0.08	13.73 ± 0.53	7.68 ± 0.14	11.72 ± 0.29*	14.19 ± 0.61	9.20 ± 0.21*	12.50 ± 0.31*
pH	9.01± 0.03	8.81 ± 0.01	8.81 ± 0.03	8.92 ± 0.05	7.91 ± 0.04*	7.87 ± 0.04*	8.86 ± 0.03	7.49 ± 0.03*	7.99 ± 0.02*
% DO	149.81 ± 1.47	133.81 ± 2.36	133.46 ± 1.25	150.81 ± 8.92	98.75 ± 0.62*	101.86 ± 1.67*	141.66 ± 2.90	108.81 ± 0.77*	110.90 ± 1.07*
Conductivity (µS cm^{-1})	0.80 ± 0.03	0.83 ± 0.03	0.82 ± 0.03	0.84 ± 0.04	0.93 ± 0.04	1.09 ± 0.17*	0.85 ± 0.03	0.98 ± 0.02	0.97 ± 0.03*
Alkalinity	48.33 ± 10.33	58.00 ± 4.35	63.00 ± 5.10	60.00 ± 6.77	63.67 ± 20.97	131.67 ± 6.93*	58.67 ± 12.84	79.67 ± 7.73	121.33 ± 4.46*
Nitrate (µg N-NO_3 l^{-1})	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00
Rotifera abundance (ind l-1)	0	15.00 ± 8.46	6.67 ± 3.33	8.33 ± 3.07	0	3.33 ± 3.33	30.00 ± 20.49	8.33 ± 8.33	0
Copepoda abundance (ind l-1)	33.33 ± 22.46	1.67 ± 1.66	13.33 ± 8.81	18.33 ± 8.72	0	0	16.67 ± 14.75	0	0
Total zooplankton (ind l^{-1})	33.33 ± 22.46	16.67 ± 8.02	20.00 ± 7.30	26.67 ± 6.66	0	3.33 ± 3.33*	46.67 ± 20.92	8.33 ± 8.33	0*
Total phytoplankton (cells l^{-1})	$17*10^4$	$98*10^3$	$79*10^3$	$27*10^3$	$35*10^2$*	$16*10^3$*	$38*10^3$	$24*10^1$*	$61*10^1$*
Small size phytoplankton (cells l-1)	$15*10^2$	$23*10^2$	$17*10^2$	$14*10^2$	$64*10^1$	$46*10^2$	$34*10^2$	$1.8*10^2$	$21*10^2$
Medium size phytoplankton (cells l-1)	$17*10^4$	$95*10^3$	$77*10^3$	$24*10^3$	$26*10^2$	$12*10^3$	$34*10^3$	$20*10^1$	$40*10^2$
High size phytoplankton (cells l-1)	$13*10^2$	$13*10^2$	$59*10^1$	$18*10^2$	$37*10^1$	$43*10^1$	$97*10^1$	$2.4*10^1$	$25*10^1$
Chl *a* (µg l-1)	1.98 ± 0.29	1.97 ± 0.40	3.94 ± 0.76	1.89 ± 0.27	0.78 ± 0.10	1.57 ± 0.14	1.25 ± 0.12	1.60 ± 0.79	2.00 ± 0.93

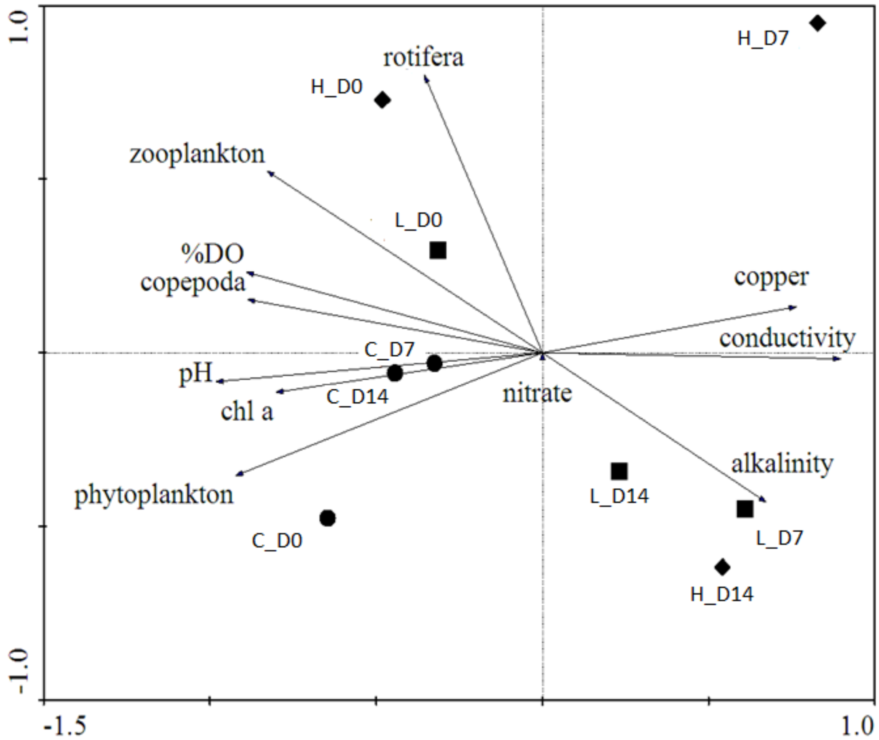
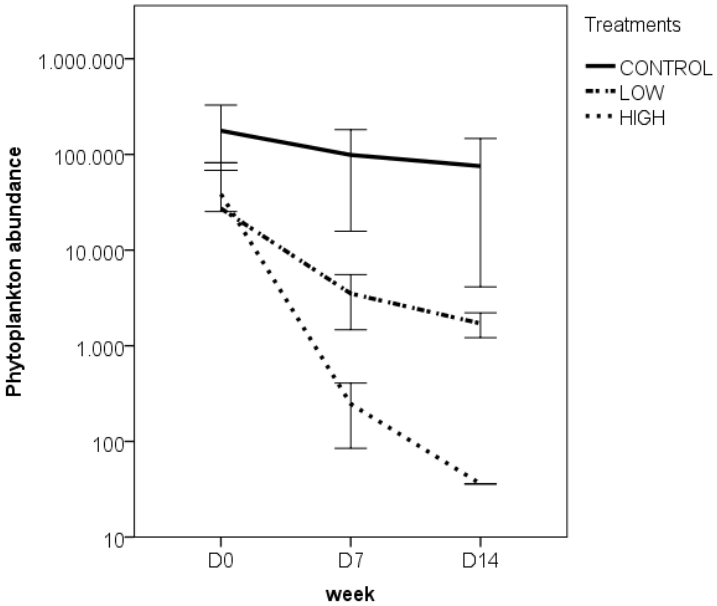


***Figure 1**. Physical-chemical PCA ordination graph. Arrows represent the lineal combination of zooplankton variables with the first and second axes. C, L and H stand for control, low and high copper treatments respectively. D indicates the sampling days.*

Phytoplankton presented differences in population abundance among microcosms (F = 5.447, P = 0.045, *Table 2*). A drastic decrease of phytoplankton populations can be observed after the copper application in the treatments with respect to the control (*Fig. 2* a). Chlorophyll *a* did not show significant differences (day 0, χ^2 = 2.648, P = 0.104; day 7, χ^2 = 2.406, P = 0.124; day 14, χ^2 = 2.351, P = 0.129;) even though it was lower in microcosms treated with copper (*Table 2*). Our functional indicator, Chl *a*, decreased but not significantly under both copper concentrations, in accordance with phytoplankton abundance decrease. In addition, variation of phytoplankton size classes of small, medium and high phytoplankton cells showed that copper treatment led to an increase in the small size group (*Fig. 2*).

Total zooplankton abundance was negatively affected mainly at the end of the experiment. The average abundance of total zooplankton during the study period was 23, 10 and 18 ind l^{-1} in the control, L and H treatments, respectively. The zooplankton community was represented by the presence of rotifera (*Euclanis sp., Brachionus sp. and Monostila sp.*) and copedoda (Calanoida). Zooplankton abundance (Table 2) showed statistical differences among the controls and treatments at the end of the experiment (χ^2 = 9.500, *P* = 0.009) and was lower in L treatment (Wilcoxon test Z = -2.060, *P* = 0.039,) and H treatment (Wilcoxon test, Z = -2.060, *P* = 0.039) than in controls. There were not statistically significant differences among zooplankton groups in control and treatments but they behaved in different ways. Copepods disappeared at the end of the experimental period, while the rotifers increased their abundance.

a)



b)

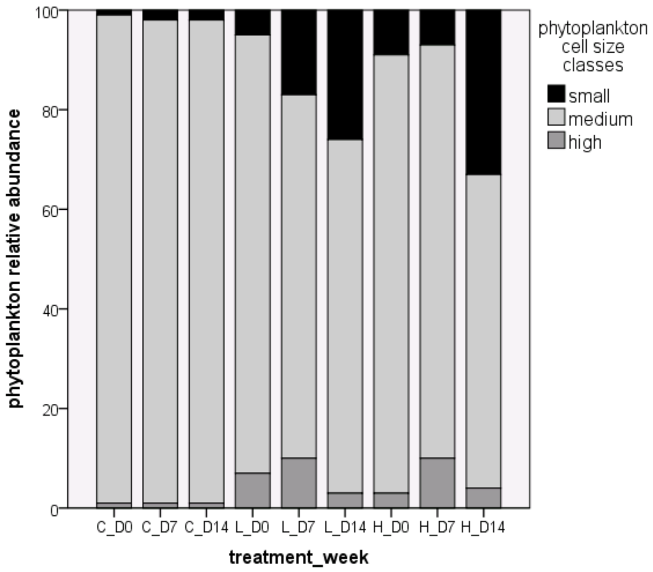


Figure 2. *a) Phytoplankton abundance (cells L-1) and, b) cell class proportion (%) along the experiment.*

Discussion

Even though there are some specific differences in physical-chemical parameters during the experiment, PCA shows that those differences were not relevant enough to discriminate among treatments. Therefore, all microcosms were under the same water quality and an environmental condition which allows us to refute that community differences are neither related to water quality and environmental-induced differences among microcosms, but owing to treatment effects.

Phytoplankton was highly affected during the whole experiment in both low and high treatments. Even working under legal copper concentrations, there are negative impacts on the aquatic community since phytoplankton abundance in treatments differed from the control abundance. Phytoplankton sensitivity to copper has been reported in other studies (Santos et al. 2002, Nayar et al. 2004). Moreover the changes in cell size group distribution showed by cytometry give information about the impairment in the plankton community and can be used as an early warning signal. Besides this fact, small size populations increased in microcosms treated with copper (Fig. 2 b). This implies a variation of predator-prey mass ratio. Body size relation is important in trophic interactions owing to its influence in growth efficiency. Growth efficiency depends on the relative body size of the prey, and on the prey density (Kerr & Dickie 2001), and copper sulfate treatments have affected both aspects. Therefore, phytoplankton cell size changes towards smaller sizes may have indirect effects upon the zooplankton community through a reduction in its growth efficiency. Phytoplankton structural features showed the first warning signals: these were a drastic decrease of phytoplankton abundance and changes towards smallest cell sizes after copper application; hence, its measure could be used as a simple and efficient tool to identify an early impairment signal. In this sense, flow cytometry has been shown as a very rapid and useful technique. As has been mentioned before, a warning signal is an important component of the integrated approaches that are needed to acquire a general knowledge of toxic impact allowing predictions and early mitigation measures (Schmitt-Jansen et al. 2008) and flow cytometry could be easily incorporated in the assessment and biomonitoring programs.

The delay in zooplankton response could be related to an indirect effect of copper on trophic interactions. The direct effect of copper on phytoplankton affected zooplankton food availability. Therefore food resources decreased for zooplankton but the effect on zooplankton was not detected right away after copper application. Moreover, this different response timing is also related with the different life span of phytoplankton and zooplankton, being faster in phytoplankton. Even though there were no statistically significant differences among zooplankton groups in control and treatments, they behave in different way. For instance, the copepoda disappearance in some treatment microcosms suggest a specific impact in that group's ability to face the experimental conditions, and consequently in its potential recovery capacity. At the same time, rotifera responded differently to copper treatments, increasing its abundance in treatment microcosms, which implies that there had been a community shift both in L and H treatments that could not be observed at total zooplankton abundance level. In fact, the control microcosms had more than double that of the zooplankton in both L and H treatments, showing the importance of analyzing changes at a lower hierarchical level in order to better understand the changes at a higher hierarchical level. Going deeper into zooplankton shift, it has been shown in other studies that rotifera are more tolerant immediately after copper application than other organisms even up to 20 mg l^{-1} of copper, however after 8 days under copper exposure from 0.5 mg l^{-1} to 20 mg l^{-1} its population was dramatically affected (Källqvic & Meadows 1978). Large-bodied zooplankton also is more sensitive to environmental stressors including pesticides than their smaller congeners (Havens & Hanazato 1993). Further, copedoda and rotifera play a different role in the ecosystems and in the food web structure. For instance, macrozooplankton, as copedoda, grazing pressure has a stronger role than rotifera in regulating phytoplankton which is an important function to control eutrophication

(Miracle et al. 2007). Kasai & Hanazato (1995), using experimental ponds, observed that the herbicide simetryn caused a decrease in zooplankton density due to indirect effects related to a decrease of algae. But picking up the changes in phytoplankton size, it is interesting to note that at the end of the experiments, both L and H treatments, showed higher proportion of small phytoplankton cells than control. The smallest filter-feeders could take advantage exploiting the mentioned small food resources, increasing their abundances. In addition, the community shift could be a response caused not only by the apparent higher rotifera tolerance to copper but also by other indirect situations. The main indirect effect is the decrease of competence for food resources due to copedoda reduction that allows the increase of rotifera population. Miracle et al. (2007) found an inverse relationship among rotifera and cyclopoida copepod abundance under perturbation. This inverse relationship of rotifera and copedoda under perturbation has been found in other studies. For instance Richard et al. (1985) observed how under herbicide treatment there were shifts from copedoda and copedoda-cladoceran dominated communities to rotifera and small cladoceran dominated communities. A similar relationship was found by Gagneten & Paggi (2009), under heavy metals treatments (Pb and Cu) rotifera increased while copedoda and cladocera decreased. Both studies used such relationship as a tool to characterize the water bodies under study: in the first case the trend towards rotifera was identified as an indicator of eutrophy impairment and in the second case as a tool to determinate heavy metals impairment. In this study, an inverse relationship between rotifera and copedoda matches with other observations that indicate copper impairment as the decrease of total zooplankton in the treatments. Therefore, it supports its use as an easy and cost-efficient indicator and warning signal of contamination in aquatic systems as Gagneten & Paggi (2009) also suggested. The negative impact that has been showed during this short term experiment on the poorest food resources could be intensified, in the long term, to the zooplankton community. The impairment in trophic relationships observed due to copper exposition, allows considering both, phytoplankton and zooplankton changes, as early warning signals. In nature, loss of species at basal trophic levels can affect production at higher levels and thus can also lead to decreased energy transfer efficiency (Gamfeldt et al. 2005). Undoubtedly, further studies must be developed to confirm these results in long term exposition, with different toxic substances, and this with holistic approaches that can detect indirect effect and alarm signals as the phytoplankton did.

Chlorophyll content is used to highlight stress due to a single environmental factor or to a combination of different environmental factors, but it also constitutes potential biomarkers of anthropogenic stress (Ferrat et al. 2003). However, in the present study Chlorophyll-*a*, as a functional indicator, has not been shown as effective as structural changes indicating alterations in plankton community.

This study works towards a deeper understanding of the agrochemicals negative effects on plankton communities at concentrations above but also below their legal limits. Surprisingly, even legal limits do not protect the plankton community. This result emphasizes the need of more ecological and realistic approaches to ensure adequate regulation limits in order to achieve a balance between development and conservation.

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CHAPTER 2

“Could a single copper sulfate pulse within legal limits change plankton community’s features?”

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Could a single copper sulfate pulse within legal limits change plankton community's features?

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ABSTRACT

Current main environmental pressures are highly linked to human population growth together with high consumption rates. One of the main consequences is an increase in food demand resulting in an increase of use of agrochemicals by intensive agriculture. This activity is causing the pollution of aquatic systems compromising ecosystem services. In order to reach protective legal limits, more ecological relevant exposures should be explored. The aim of the work was to study the effects of agrochemical concentrations within legal limits on a planktonic community. An experiment using a non-target aquatic community was done to test the hypothesis of negative effect of a single pulse of copper sulfate within legal limits on plankton abundance, structure, richness and diversity endpoints. The microcosms (20Lvolume) were established during 21 days, using six replicates for controls (C) and for two concentrations of copper sulfate (High treatment, H: 20 µg Cu L^{-1}; and Low treatment, L: 2µg Cu L^{-1}), both within copper legal limit following the Water Framework Directive. General Lineal Model found significant differences at phytoplankton abundance endpoint. The Principal Response Curve of zooplankton pointed differences on abundance and structure between treatments and controls, what indicates trends of community changes owing to copper sulfate effects. In conclusion, even if copper concentrations under study were within legal limits they have shown the potential to induce changes on planktonic community's features.

Key works: plankton, copper, PRC, microcosms

INTRODUCTION

Mediterranean aquatic systems are one of the most altered natural systems owing to agricultural practices worldwide (Casado and Montes, 1995; Beja and Alcazar, 2003; Parra *et al*., 2005; Zacharias and Zamparas, 2010; García-Muñoz *et al*., 2010). Their catchment: lake ratio is lower than in temperate lakes, so they experience stronger catchment effects (Álvarez-Cobelas *et al*., 2005), many of them related to the intensive agriculture management (Guerrero *et al*., 2006). Although the socio-economic value of agriculture cannot be denied, its integration with more realistic ecological risk assessment must be a priority to prevent environmental hazard and consequently, human ones. The Water Framework Directive (WFD) faces this concern in relation to water bodies, so that it intends to assess, monitor and manage the ecological and chemical status of all surface and groundwater bodies.However, often-freshwater systems are ignored and undervalued (Downing, 2010). This preconception should change in light that freshwater systems, from large lakes to small ponds, contribute both to regional diversity (Oertli *et al*., 2002; Downing, 2010; Gilbert *et al*., in press) and to global cycles playing an important role, for instance, in carbon cycling (Downing, 2010).

In this study context, aquatic systems can be impacted by agrochemicals as copper sulfate ($CuSO_4$) that is used to control the fungi *Cycloconium oleaginum* in olive tree cultivation. Even though copper can be found naturally in different forms, it can be toxic in aquatic systems as Cu^{2+} (Lenwood*et al*., 1998). Water Quality Criteria (WQC), following the European WFD, establishes a maximum legal limit in waters of 40µg $L^{-1}$of copper. However, copper WQC may not be safe enough for not-target species since it is mainly based on single test organisms, but little information about the effects on other taxa is used in the regulation process. In this sense, previous studies has also found that 40µg $L^{-1}$of copper is not a safe limit for amphibians as *Bufo bufo, B. calamita* and *Pelodytes ibericus* (García-Muñoz *et al*.,

2011) or for plankton community (Del Arco *et al.*,*in press*); neither for marine fishes as *Sparu saurata* (Oliva *et al.,* 2007).These impacts have consequences at different hierarchical levels, from the individual by morphological, physiological and biochemical alterations, to the community level through the loss of diversity, and impairing the value and services that healthy ecosystems provide (Montes and Sala, 2007). So then, community studies aiming at increase the knowledge about toxicant effect within legal limits on a wider range of species should be encourage in order to detect sublethal, direct and indirect effect upon the aquatic systems.

The present study is the second of a series of experiment aiming to assess the effect of copper sulfate on plankton communities, both above and below legal limits. In the first study, we explored copper concentrations of 200 and 20 µg L^{-1}, and adirect negative effect on the plankton community structure was found (Del Arco *et al.*, in press). Therefore, a further step is to understand how even legal lower copper concentrations would affect the community. So then, the present experiment was proposed to study copper sulfate pulses under 20 µg L^{-1}. In this framework, the hypothesis of this study was that treatments within legal limits would affect the aquatic community owing to both, sub lethal and indirect effect that are not detected in single species test used for legislation purposes, raising a concern about long term effects.

MATERIAL AND METHODS

Microcosms

Eighteen outdoor microcosms (n = 6, circular plastic bucket of 20 L volume) were set based on and adapted protocol from OECD (2006). Microcosms were filled with 18 liters of water and 5 cm of sediment. Filtered water came from a supply artificial pond (HUMEXPUJA, experimental wetland infrastructure in the University of Jaén, Spain). Homogenized sediment came from a natural freshwater wetland [Casillas wetland, UTM 30SVG1083 with a surface area of 2.7 ha (Ortega *et al.*, 2006)]. Microcosms experiment was developed during 3 months, from March to May 2012. Previously to the start of the experiment, astabilization period of 7 weeks was done in order to allow the development of the plankton and benthic communities from resistant structures present in the sediment.In addition, there was a pre-treatment week (D0) with no treatments before the copper pulse (D7).

Copper analysis

Microcosms were exposed to 2 concentrations of copper sulfate, High treatment (H): 20µg CuL^{-1}; and Low treatment (L): 2µg Cu L^{-1}. Following the WFD, the Spanish national legislation establishes a copper WQC level of 40µg Cu L^{-1}(BOE, 2011). Therefore, both treatments, H and L, fall within legal limits.

Six replicates were used in control and both treatments. Nominal dosages of copper sulfate were added directly spiked over the water surface on the microcosms as an only pulse on day 7 (D7) for the whole experiment period. After stirring, water samples were taken to perform direct analysis by Inductively Coupled Plasma (ICP) Mass Spectrometry in order to confirm the target nominal concentrations of copper. In addition, water samples from the controls were analyzed to ensure no copper was present.

Physical chemical variables

Each microcosm was surveyed four times,on days 0, 7, 14 and 21 (D0, D7, D14 and D21), after the 7 weeks of stabilisation period. Data on physical-chemical variables: temperature, pH, dissolved oxygen and conductivity,were obtained using a field probe (YSI-556 MPS). At the same time water samples (100 mL) were taken and transported in cold and darkness conditions to the laboratory for the analysis of alkalinity. Alkalinity was measured using an 848 Tritino Plus devise.

Phytoplankton and zooplankton endpoints

Phytoplankton and zooplankton were sampled weekly. Phytoplankton samples (4 mL) were collected with a pipette after homogenization by gently stirring the microcosm. Samples were preserved with glutaraldehide (2% f.c.), frozen in liquid nitrogen and stored at - 80ºC until the evaluation of their abundance and size distribution with a BD- LSR Fortessa flow cytometer. The transformation of the forward scattering signal (FSC) of the flow cytometer to cell volume was carried out by means of a calibration curve. This function was obtained from the analysis of 5 sets of calibration beads ranging from 1 to 11 µm. Phytoplanktonic groups were established according to 4 size ranges <2 µm; 2-8 µm; 8-20 µm and >20 µm ESD (named as pico-, ultra- and nano -). Integrated zooplankton samples (500 mL) were taken with plankton net (mesh size of 60 µm) and preserved in situ with buffered formaldehyde (4% f.c.). The filtered water was returned to the microcosm. Zooplankton was identified into different Taxonomic Practical Levels (TPL) (Van Wijngaarden *et al*., 2005): Ostracoda, Copepoda, Cladocera and Rotifers. Moreover copepods were divided in two groups, nauplii (that include together calanoids and ciclopoids) and adults plus copepodites. The cladocera were integrated by Ceriodaphnia sp., Alona sp. and Macrotrix sp. The abundance and richness of each TPL and the biodiversity (modified Shannon-Wiener diversity index: $H' = \sum p_i \log_2 p_i$, the modification refers to the use of TPL instead of genera of species) in all experiment were evaluated.

Data analysis

GeneralizedLinear Models analysis (GLM) was used for test differences between treatment and controls for all physical-chemical variables and taxon levels.Prior to analysis, data were tested for normality and homoscedasticity using the test of Shapiro-Wilk and Levene respectively. Abundance data of zooplankton and phytoplankton were log (x+1) transformed. The analyses were carried out with the SPSS 19 computer program.

Moreover, in order to evaluate the community response to treatments, a Principal Response Curve (PRC) analysis was done using CANOCO software package, version 4.5 (Van den Brink and ter Braak, 1999).PRC is a multivariate technique recommended to analyse complex changes in community structure over time under a treatment exposure in micro/mesocosms experiments (European Commission, 2002; Sanderson *et al.,* 2009). It is based on the abundance response of TPL along the experiment in each treatment. The null hypothesis implies that the PRC analysis does not show the treatment effects on the community (Frampton *et al.*, 2000).

RESULTS

Copper concentrations

Average Exposure Concentrations (AEC) fit target nominal copper concentrations of 2.63 ± 0.83 µg L^{-1} for L treatment, and 28.10 ± 2.46µg $L^{-1}$for H treatment.

Physical-chemicaldata

Physical-chemical average values along the experiment were highly similar among all microcosms (Table 1). GLM (Univariate analysis) of physical-chemical parameters did not showed significant differences between control and treatments and neither between treatments (p>0.05).

Table 1. Physical and chemical measurements (mean ± SE) of controls and treatments along the experiment.

Week	W0			W1			W2			W3		
Treatment/ Variable	C	L	H	C	L	H	C	L	H	C	L	H
Temperature (ºC)	13.14 ± 0.23	12.93 ± 0.39	12.88 ± 0.47	15.78 ± 0.04	15.72 ± 0.07	15.77 ± 0.09	16.23 ± 0.07	16.13 ± 0.13	16.32 ± 0.17	16.02 ± 0.14	15.95 ± 0.09	15.95 ± 0.11
pH	7.84 ± 0.14	8.04 ± 0.13	7.98 ± 0.32	7.74 ± 0.29	7.66 ± 0.21	7.80 ± 0.21	7.51 ± 0.21	7.21 ± 0.16	7.30 ± 0.20	7.47 ± 0.18	7.79 ± 0.45	7.90 ± 0.26
% DO	87.66 ± 2.88	87.73 ± 2.64	88.72 ± 2.52	71.94 ± 4.71	72.43 ± 2.42	72.42 ± 1.65	71.87 ± 3.84	72.43 ± 2.42	72.42 ± 1.65	81.62 ± 3.34	76.07 ± 6.35	84.58 ± 3.33
Conductivity (µS/cm)	790.20 ± 47.96	747.83 ± 36.55	779.5 ± 73.80	902.40 ± 27.81	822.83 ± 45.16	848.83 ± 81.95	874.48 ± 157.89	889.33 ± 117.97	979.33 ± 87.16	849.33 ± 32.54	767.17 ± 42.00.	771.00 ± 82.73
Alkalinity (mg $CaCO_3$ L^{-1})	265.60 ± 190.11	104.67 ± 19.98	93.00 ± 14.24	62.00 ± 10.51	67.00 ± 4.64	77.00 ± 35.46	81.00 ± 6.81	65.33 ± 5.26	70.33 ± 13.06	49.33 ± 11.06	26.00 ± 11.84	44.67 ± 16.25

Biological data

Figure 1 shows the phytoplankton abundance dynamics along the experiment.GLM shows that there is a significant difference in the last week (D21) in terms of phytoplankton abundance means values between H, L and C treatments (F = 36.403, p< 0.001) (Figure 1, Table 2). A post hocTukey test denotes that the abundances of phytoplankton in L treatments were different to controls and H treatments. However, no differences were detected in chlorophyll-*a* concentrations or in phytoplankton size classes (Figure 2).

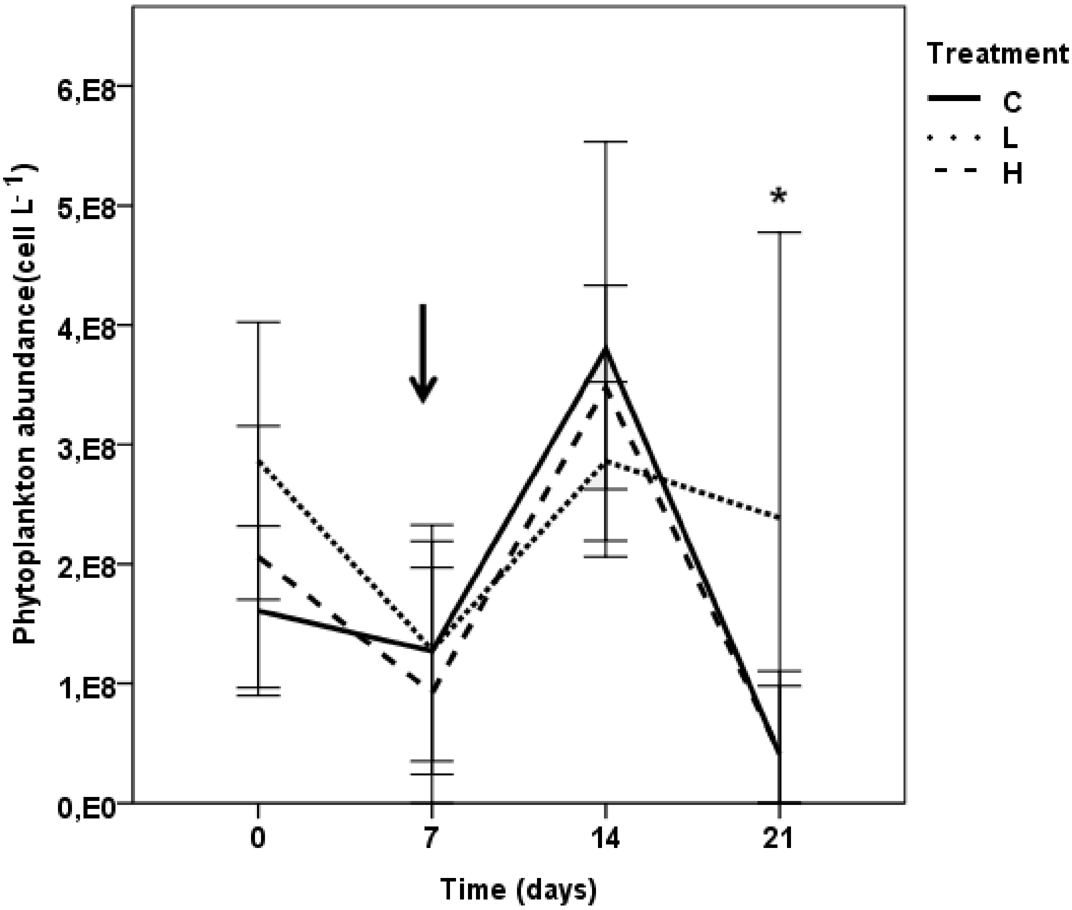

Figure 1. Phytoplankton abundance dynamics along the experiment.* Denotes statistically significant differences with the controls. The arrow indicates the chemical pulse.

Table 2. Phytoplankton abundance, Chl*a* concentration,zooplankton abundance, taxa group abundance, richness and diversity measurements (mean ± SE) of controls and treatments by the end of the experiment (D21).Arrows indicate the negative or positive abundance change as the copper concentration increases respect to the controls and hyphen indicates that there was not a clear dose-response.

Endpoints	Control	Low	High
Phytoplankton (ind mL^{-1})	6756 ± 3413	70137 ± 45859	51668 ± 46456
Picophytoplankton (ind mL^{-1})	6525 ± 3414	67905 ± 45422	51281 ± 46192
Ultraphytoplankton (ind mL^{-1})	137 ± 54	1706 ± 783	356 ± 264
Nanophytoplankton (ind mL^{-1})	94 ± 51	525 ± 469	31 ± 13
Chl a (µg L^{-1})	4.76 ± 1.12	2.70 ± 0.24	5.89 ± 1.92
Zooplankton (ind L^{-1})	769 ± 191	562 ± 106	410 ± 85
Rotifers (ind L^{-1})	756± 190	548 ± 108	398± 83
Cladocera(ind L^{-1})	0.80 ± 0.37	3.00 ± 2.05	1.60 ± 0.81
Copepoda(ind L^{-1})	3.80 ± 1.24	4.33 ± 1.33	7.00 ± 3.21
Nauplii(ind L^{-1})	7.60 ± 2.71	6.33 ± 1.41	2.80 ± 1.02
Ostracoda(ind L^{-1})	0	1.17 ± 0.65	0.20 ± 0.20
Modified Shannon-Wiener	0.15	0.24	0.36
TPL Richness	3	4	4

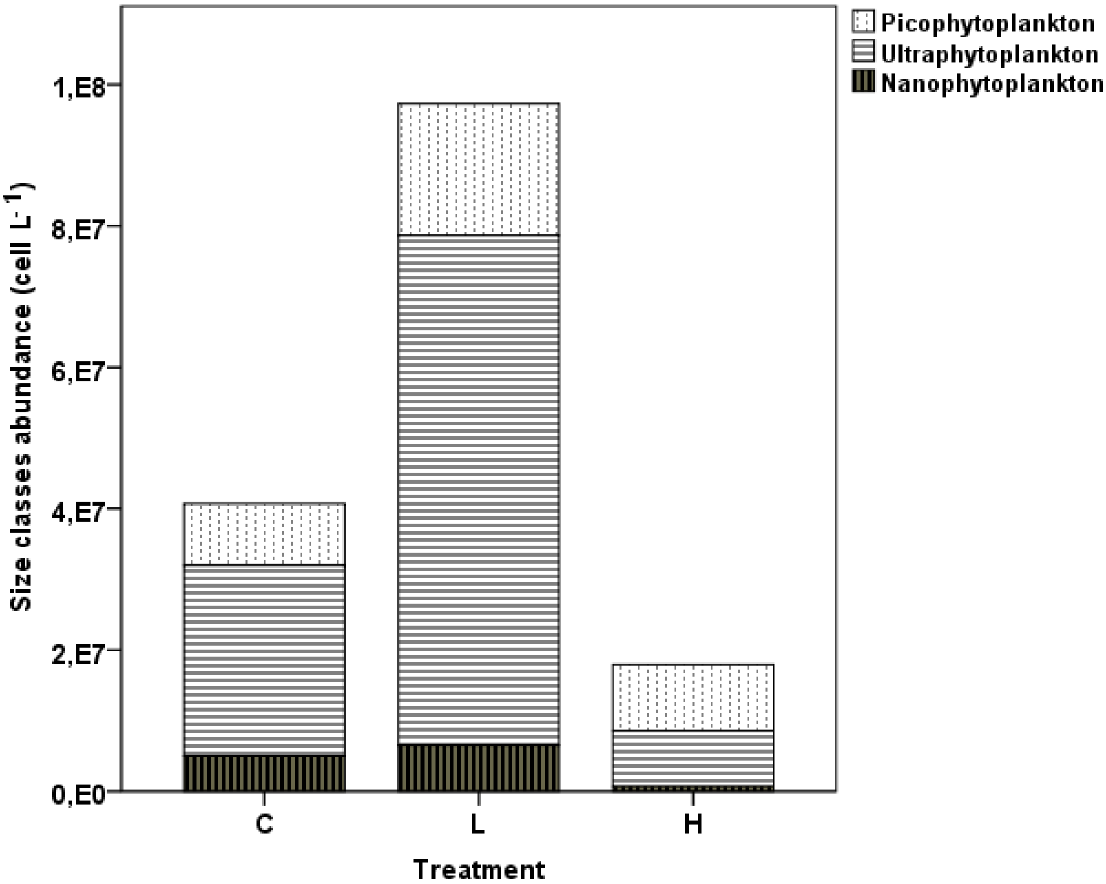

Figure 2. Phytoplankton abundance of size classes (pico, ultra and nanoplytoplankton) at the end of the experiment (D21).

Zooplankton abundance and community structure from the sediment did not present statistical significant differences on D0 before exposure so then any differences after the exposure would be related to the treatments. TPL Richness and diversity (modified Shannon-Wiener) values were not statistically different among controls and treatments neither along nor at the end of the experiment even though total abundance decreased as concentrations increases (Table 2). PRC diagram (Figure 3) shows the overall response of community structure and TPL abundance changes owing to each treatment along the experiment. 1D-plots represent the weight of the TPL in the overall response of the community: positive TPL weight mean that the TPL are likely to follow the PRC response, negative TPL weight show the opposite trend;

and, the response of TPL weight near cero is not related to the main response shown by PRC (Van den Brink and ter Braak, 1999).

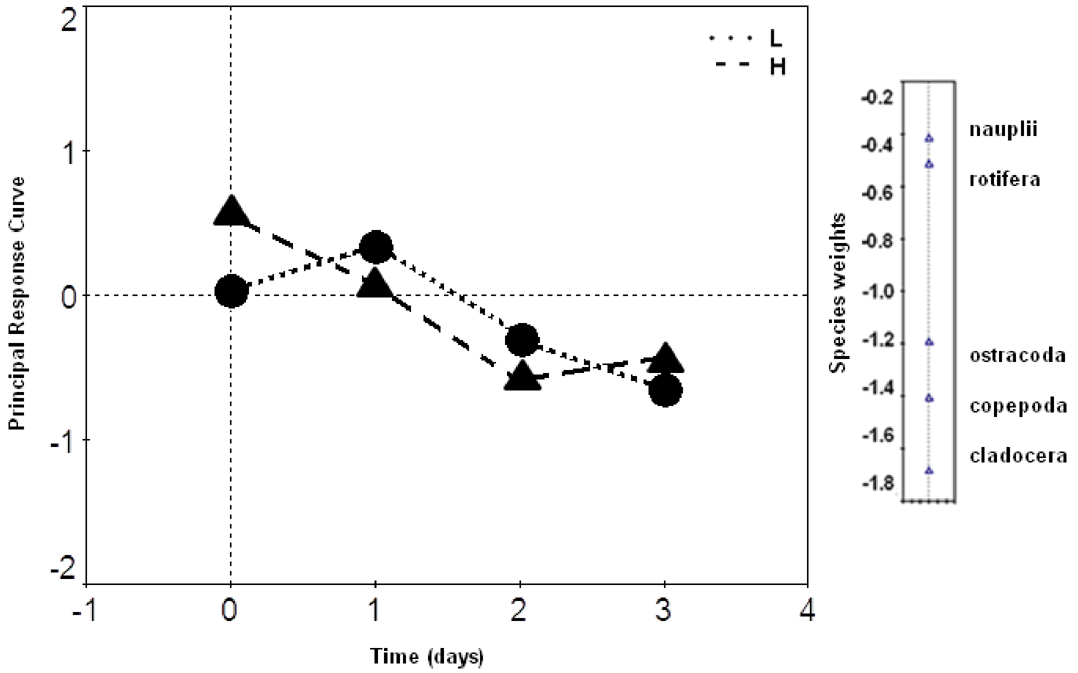

Figure 3. Principal Response Curve (PRC). On the left, ordination method represents the main community respond to the treatment effect over time respect to the controls. The graph summarizes the zooplankton community respond based on TPL (Rotifera, Copepoda, Nauplii, Cladocera and Ostracoda). On the right, the 1-D plot the species weights what represent the level of affinity that each taxa have with the main trend of the PRC.

DISCUSSION

The experimental design attending to expose microcosms to equal natural environmental variability with respect to outdoor environmental condition was achieve since there were not statistical significant differences in physical-chemical variables, consequently the copper treatments were the only discriminating factor.

Phytoplankton and its size distribution are recognized as an important ecological attribute of aquatic ecosystems (Guerrero and Castro, 1997). The results obtained in this study showed that significant statistical differences were found in phytoplankton abundance at the end of the experiment (D21), with an increase of phytoplankton abundance in L treatment, principally in the ultraphytoplankton size class. It is important also to note the quasi-extinction of the largest size class (nanoplankton) in H treatment (Figure 2). This is a typical characteristic of perturbed ecosystems, the general pattern of reduced size in stressed communities (Kerr, 1974) that in our case is developed towards an increase in the pico and ultraphytoplankton size class and a reduction in the abundance of the nanoplankton. Similar results are obtained in the bibliography, indicating the effects of a wide range of abiotic factors on the relative contribution of nanoplankton (Reynolds, 1984; Rojo and Rodríguez, 1994). By contrast, chlorophyll-a concentrations do not showed statistical differences between control and treatments ($p>0.05$). It is well known the important role of the nanophytoplankton in the chlorophyll-a concentration in many aquatic systems, with values over 50% of the total chlorophyll-a concentration (Rodríguez and Guerrero, 1994). As nanophytoplankton is the size class that more variation presents in the experiment between treatments, it was expected to obtain statistical differences between them. However, there were not statistically significant differences despite the lower chlorophyll-a concentration in the L treatment respect to C and H treatments (Table 2).

Zooplankton abundance and the community structure (Figure 4, PRC) do not showed significant statistical differences between control and treatments. Despite the absent of significant statistical differences of the PRC ($F=2.259$, p-value = 0.9620), it amplify the information that zooplankton abundance dynamics show because it gives information about TPL instead of total zooplankton abundance what can detect taxa shifts. Similar results were obtained by Hillis *et al.* (2007) when studied the effects of the antibiotic Monensin on zooplankton communities in aquatic microcosms. Even though the PRC was not significant, it was considered obvious a negative effect of the antibiotic at the greatest treatment concentration. In the present study, the PRC analysis showed a tendency of the treatments towards a different community structure that is related with observed changes at TPL endpoints as abundance, richness and diversity (see Table 2). The abundance of rotifers and nauplii decreased as the copper treatment concentration increased; while copepods, cladocera and ostracoda increased (Table 2). Those three TPL mark the differences on community structure, richness and diversity (modified Shannon-Wiener) between controls and treatments. Surprisingly, both richness and diversity are higher in the treatment than in the controls at the end of the experiment (see Table 1). This kind of response agrees with the intermediate disturbance hypothesis (Connell, 1978; Menge and Sutherland, 1987; Hanazato, 2001) and with the fact that the exposure concentrations are within legal limits so then the impact is not drastic but still there is an effect of copper concentrations. This community shift could be related to food edibility. The above-mentioned reduction in the nanophytoplankton size class in H treatment coincides with the lower abundance of rotifers and nauplii compare to C and L treatment. It may indicate a community structure change related to grazing pressure disruption. Therefore, long term effect owing to indirect effect impacting trophic relationship could be discussed as the need to have prolonged the experiment to be able to catch such potential effects. Indirect grazing pressure disruptions have been found in previous mesocosms studies (Van den Brink *et al*., 2000; Stampfli, 2011). For instance, Van den Brink et al. (2000) studied carbendazim effect in zooplankton and phytoplankton communities and found an increase of phytoplankton owing to a reduced grazing pressure from zooplankton depletion by the fungicide. It indicates that the addition of one pollutant may cause indirect top-down effects (Baird and Burton, 2001) that cannot be detected in single species test and short-term studies. Changes in the abundance of rotifers and nauplii mark the decrease of zooplankton in the treatments, while cladocera and copepod abundance is higher in the treatments. It contradicts the most common general order of sensitivity from lower to higher sensitivity of rotifers > copepods > cladocera (Hanazato, 1998; Relyea, 2005). It also leads to think in an indirect effect related to changes in phytoplankton structure and grazing pressure that may temporarily favor the most sensitive taxa to the toxicant. For instance, Gui and Grant (2008) studied the combined effect of food availability and chemical exposure on *Drosophila melanogaster*, and conclude that a release from competition on food resources could counterbalance the chemical impact. This community structure disruption may intensify over time. Therefore, a zooplankton decrease may follow two main paths in a long term: a) it may recover and get similar to control supporting that copper cause not effect as GLM and PRC suggested owing to its lack of significance, and b) it may keep a decreasing tendency suggesting copper long term effect related to community abundance and structure changes. A longer experimental period would have helped to discern among those two potential paths. A post-treatment of 21 days could not have been enough to capture the effect on the complete life cycle of the organisms; nevertheless it has been enough to warn about agrochemical induced changes.

Taking into account the richness and diversity index, the slightly increase in the treatments highlighting the importance of direct and indirect effects. Increases of diversity after a chemical pulse have been previously observed. For instance, Hanazato (1997) reported a species richness increase in ponds treated with insecticides as a consequence of competition interactions alterations. In our experiment, the increase of diversity is mostly linked to the survival of ostracoda and the better performance of copepoda and cladocera potentially related to a release of competition pressure for the decrease of other dominant competitors taxa in the treatments as rotifers and nauplii. It may suggest that at taxa levels to face perturbations, apart from the initial diversity, also play an important role the indirect effects (in this case, changes in phytoplankton size classes abundance), the intensity of perturbation and timing of population and ecosystem responses (Downing and Leibold, 2010). The diversity-stability debate complexity overcome our short-term data, however, it was considered important to include this thought. Such importance comes from the fact that all politic–science meeting end with the goal of preserves diversity (Joint EEA-JRC-WHO REPORT, 2008). Therefore, studies should attend to contribute to a better understanding of this ecological term to develop adequate policies and realistic data set of acceptable agrochemical thresholds.

The results of the first experiment of this series of studies (Del Arco *et al.*, in press) agree with the current results. In both experiments, concentrations within legal limits of 2 µg Cu L^{-1} were explored. There are two main common changes: a) a zooplankton community change together with a decrease of abundance as copper concentration increases, and b) a phytoplankton community change towards small size classes. A direct comparison of both experiments is inappropriate owing to differences in seasonality and community structure. However, the identification of common patterns is a remarkable fact.

Considering our hypotheses**,** even treatments within legal limits could negatively affect the aquatic community; that it is observed in phytoplankton community. The zooplankton community has been negativelyaffected at structural levels suffering a shift tendency in community composition and an abundance decrease was clearly visible in the H treatments. Impacts in L treatments are less detectable but raising concern about long term effects prevention capacity. In addition, as expected, indirect effect have taken place as phytoplankton community structure reflected; phytoplankton abundance increased owing to low herbivore pressure what highlight the importance of more complex test than single species ones to be able to detect these indirect impacts on the community structural changes

CONCLUSIONS

Legal concentrations of toxic substances in aquatic systems are establishes based on single species test using standard species, little information is known about how such legal concentrations will affect the rest of the taxa in more complex ecological scenarios. This study highlights that even a single copper sulfate pulse within WQC would have negative impactsupon plankton aquatic community.

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CHAPTER 3

"Effects of nitrate concentrations within legal limits on natural assemblages of plankton communities"

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Effects of nitrate concentration within legal limits on natural assemblages of plankton communities

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ABSTRACT

Intensive agriculture is the leading pertubation on freshwater systems due to agrochemical imputs that compromise biodiversity and ecosystem services. Agriculture importance cannot be neglected neither the fact that freshwater biodiversity is being depleted worlwide owing to anthropic pressures. Therefore, a balance between economic and environmental values must be achieved. The present experiment explores agrochemical (nitrate used as fertilizer) legal limits concentrations to investigate if even assumed safe limits impact freshwater ecosystems. The purpose is to assess direct and indirect effect of legal toxicant concentrations on the ecological integrity of plankton communities. This information seeks to assist policy makers with more ecological relevant results to establish legal limit concentrations that neither over- nor underestimated environmental risks. Microcosms experiments were set up outdoors with local assemblages of plankton for 8 weeks. Two nitrate treatments (n = 5) plus controls (C) were added: 1) Low treatment (L) of 25 mg l^{-1} of nitrate, 2) High treatment (H) of 50 mg l^{-1} of nitrate. In conclusion we detect first that zooplankton taxa shifted from a cladocera-dominated to a copepod-dominated community as an indirect response of phytoplankton abundance decrease as a result of nitrate exposure, and second, both L and H nitrate treatments had a negative effect on the plankton community despite of being within assumed safe legal limits.

Keywords: legal limits, nitrate, local assemblages, microcosms

INTRODUCTION

Nitrates are naturally present in aquatic systems. However, anthropic sources as fertilization in agriculture have substantially increased its natural concentrations leading to negative effects on aquatic ecosystems (Kratzer and Brezonik, 1981; Camargo and Alonso, 2006; Miracle et al., 2007). The Water Framework Directive (WFD) aims to achieve a good status of European water bodies throught a balance between environmental protection and agriculture (Directive 2000/60/CE).

An increase of inorganic nitrogen generates adverse effects on aquatic organisms (Camargo and Alonso, 2006). Single species test are the widespread methods to assess Ecological Risk Assessment (ERA). Though, there are many studies that debate the lack of ecological realisms of the single species test used for ERA (Wootton, 2002; Brooks et al., 2009; Van den Brink, 2013). The main criticisms are related with the use of standard species that may not represent the sensibility of all taxa (Cuppen et al., 2000); the lack of ecological interactions (i.e. predation and competition) (De Laender and Janssen, 2013); and the absence of studies with agrochemical concentrations commonly found in aquatic ecosystems (LeBlanc et al., 2012). Accordingly with these critiscms, studies with local species have shown negative impacts of nitrate upon local freshwwater invertebrates and amphibians (Camargo and Ward, 1992; García-Muñoz et al., 2011 a, b, c). For instance, Camargo and Ward (1992) exposed non-standard freshwater invertebrates (Trichoptera: Hydropsychidae) to sodium nitrate finding behavioural alterations, physiological changes and lower LC_{50} than those reported for standard fish species used in ERA. In addition, sublethal effects are also important, García-Muñoz and co-workers (2011b) studied the larval escape behavior of three anuran amphibians (*Bufo bufo*, *Epidalea calamita* and *Pelodytes ibericus*) and reported the adverse effect of nitrate on efficiency of escape.

Another important question could be made about the current legal limits of agrochemical concentrations in aquatic ecosystems, raising the question if current legal limits are safe enough (Camargo et al., 2005; Del Arco et al., 2014). Within this context and focusing on nitrate, to our knowledge, there are no previous studies to this one assessing the effect at community levels using concentrations within legal limits of nitrates. So then, this study seeks to overcome the limitations of single species tests, through studying nitrate exposure effect on more complex systems to obtain a more holistic information about the effect into the ecosystem. The experiment was design to test the hypothesis that nitrate pulses under legal limits will have negative impact on the plankton community.

MATERIALS AND METHODS

Microcosms set up

The response of plankton community to nitrate exposures whithin legal limits were explored experimentally using a simple-throphic community stablished in microcosms. Fithteen microcosms were stablished based on an adapted protocol from OECD (2006). Microcoms of 50 liters volume and filled with 45 liters of mineralized water were located outdoor in an experimental wetland infrastructure at the University of Jaén. Microcosms were inoculated with plankton species and sediment coming from a local wetland [Casillas wetland, UTM 30SVG1084 with a surface area of 2.2 ha. (Ortega et al. 2003)]. Plankton samples were collected with a vertical haul with a plankton net (53 µm). Samples were homogenized and equally distributed among the microcosms.

Sediment was extracted from the superficial layer of the wetland, homogenized and distributed as a 5 cm layer for all microcosms. Rotifers, cladocerans (*Alona* sp., *Ceriodaphnia reticulata*, and *Macrothrix hirsuticornis*), calanoid copepod (*Neolovenula alluaudi*), cyclopoids copepods (*Acanthocyclops* sp. plus *Metacyclops* sp.) and ostracods were present in the plankton samples and/or developed from the sediment resistant eggs. Phytoplankton community was expected to develop from the water inoculation together with zooplankton and sediment. The use of local species naturally occuring in local aquatic systems was considered extremelly important to catch a wider range of sensitivity than standard species may not do.

Microcosms were established in November 2012 and the experiment was finished in February 2013. There was a stabilization period of 7 weeks after the innoculation and before adding nitrate in order to favour the acclimatation and development of the plankton community. The experiment lasted 49 days, with a single fertilizer spike on day 0 There were 5 controls (C) and 2 treatments with 5 replicates as well. Ammoniun nitrate (NH_4NO_3) was added in order to achieve the following nominal nitrate concentrations in each treatment: Low (L) nitrate concentrations with 25 mg l^{-1} of nitrate, and High (H) nitrate concentrations with 50 mg l^{-1} of nitrate. Nitrate concentrations were selected based on legal limits established by the Council directive 80/778/EEC (revised as Council Directive 98/83/EEC) for nitrates and the Council Directive 91/676/EEC for nitrates.

Physical chemical variables

Physical-chemical variables, temperature, pH, dissolved oxygen and conductivity were weekly measured (D0, D7, D14, D21, D28, D35, D42, D49) using a field probe (YSI-

556 MPS). In addition, water samples (100 ml) were taken and transported in cold and darkness conditions to the laboratory for the analysis of alkalinity (848 Titrino Plus devise).

Nitrate stock solutions were prepared using ammoniun nitrate. The two nominal concentration of nitrate were aliquots of the stock solutions and spike on every treatment as a single pulse on D0 after the sampling by applying it to the water surface and gently stirring to ensure an homogeneous distribution over the water column. After stirring, 50 ml of water were taken to perform analysis of nitrate concentration to confirm the nominal concentrations. Lately, nitrate analysis was done weekly using a laboratory standart protocol based on ultraviolet methods (Standard method APHA, 1995).

Biological endpoints

Water samples where also weekly taken to evaluated chlorophyll-*a* (Chl *a*) concentration and phytoplankton community response to the toxicant using flow cytometry. Chlorophyll-*a* concentration was calculated using a previously obtained calibration curve determinate by fluorometry. Samples were filtered through Whatman GF/C glass microfibre filters, and extracted in 90% acetone for 24 h at 4 ºC. For cytometry analysis, water samples were preserved in glutaraldehide (2 %), frozen in liquid nitrogen and stored at -80 ºC until the analysis with a BD- LSR Fortessa flow cytometer. Calibration spheres were used to obtain a cell size regression curve: $y = 0.008\ x – 3.93$ (“x” = the mean of the Forward Scatter (FSC), and “y” = cell size of the cells in μm^3). Four cell size populations were established: picophytoplankton (0.4 – 2 μm^3), ultraphytoplankton (2 - 8 μm^3), nanophytoplankton (8 – 20 μm^3) and microphytoplankton (> 20 μm^3). Samples were analyzed and recorded during a time of 180 s at a rate of 60 μl min^{-1}. These data were analyzed with FACSDIVA software. The endpoints evaluated were abundance (cell l^{-1}) and community size structure (pico-, ultra-, nano- and microphytoplankton cell size classes, PCA).

Zooplankton integrated water samples (500 ml) were taken weekly from each microcosms, filtered with a plankton net of 60 μm and preserved in formaldehyde (4% f.c.). The filtered water was returned to the microcosm. Zooplankton was counted, identified and grouped to the following eight taxonomical practical level (TPL) (Van Wijngaarden et al., 2005; Del Arco et al., 2014): ostracoda order, calanoid copepod (*Neolovenulla alluaudi*), cyclopoid copepods (*Acantocyclops* sp. plus *Metacyclops* sp.), nauplii (calanoida plus cyclopoida), *Ceriodaphnia reticulata*, *Alona* sp., *Macrothrix hirsuticornis* and rotifers order. The endpoints assessed were abundance (ind l^{-1}), community structure (PRC), diversity (Shannon-Wiener diversity index) and richness. All endpoints were calculated base on TPL.

Oxygen production was estimated by diurnal oxygen fluctuations as a proxy of ecosystem productivity (Lind, 1979; Cole and Pace, 2000; Downing and Leibold, 2010; Del Arco et al., 2014). It was weekly measured at the start (8:00 am) and at the end (18:00 pm) of the autumn-winter photoperiod (10:14) using a field probe (YSI-556 MPS).

Litter decomposition was assessed incubating alder leaves (*Alnus glutinosa*) to compare the percentage of Ash Free Dry Mass (AFDM) by the end of the experiment. One litter bag for microcosms (initially containing 3 g dry weight of alder leaves) was incubated for the whole experimental period (49 days). Litter bags were 10 x10 cm and 1 cm mesh size. After retrieval, litter was rinsed with tap water, oven dried (105 ºC for 24h), weighed, ignited (550 ºC for 4h) and reweighed to determine AFDM remaining (Gessner and Chauvet, 1994).

Statistical analysis

Nitrate effects on planktonic community based on comparison between controls and treatments were assessed through analysis of variance by general lineal model (GLM) followed by Tukey test, with SPSS software. Prior to analysis, plankton data were log (x +1) transformed to meet homocedasticity and normality asumptions. In addition, a Principal Component Analysis (PCA) for phytoplankton and a Principal Response Curves (PRC) for zooplankton were done by CANOCO v4.5 software to explore community response to the treatments. Both techniques are based on redundancy analysis ordination (Van den Brink and Ter Braak, 1999). Phytoplankton analysis with the PCA aimed at identifying phytoplankton community structure response to the treatments based on cell size changes respect to the controls. A phytoplankton PCA instead of a PRC was performed because cell size classes endpoints were only 4 classes what is too little for a PRC. PRC results in a diagram showing the principal response of the community (y- axis) for all sampling days (x-axis) by plotting the deviations in time of the treatments versus the controls. The species weights (1-D plot) indicated the affinity of the species with the principal community response: positive weight means a positive correlations with the main community response; negative weight display a negative correlation, and weight close to cero means no response or very dissimilar to the main response.

RESULTS

Physical chemical variables

Tha analysis of physico-chemical variables along the experiment showed no significant differences in temperature ($p = 0.189$), pH ($p = 0.916$) and alkalinity ($p = 0.067$) in all microcosms. These variables showed average values of 8.6 ºC, 7.6 and 11.9 mg $CaCO_3$ l^{-1} respectively. By contrast, conductivity ($p < 0.001$) and oxygen ($p < 0.001$) showed statistically significant differences between the controls and treatments along the experiment after nitrate addition. Conductivity was higher and statistically different between C (260.73 ± 9.88) and both treatments, L treatment (337.37 ± 15.24) and H treatment (408.10 ± 13.90) after the spiking of nitrates. Dissolved oxygen also differed between C and H treatment as well as between L and H treatments in the two last sampling days. The average oxygen concentrations along the experiment were: 8.41 ± 0.09, 8.34 ± 0.09 and 7.88 ± 0.08 mg l^{-1} in C, L and H treatments respectively.

Nitrate measurement after spiking reveals higher concentrations than target nominal concentration: 51.80 ± 7.88 mg l^{-1} in L treatments and 98.41 ± 38.19 mg l^{-1} in H treatments. However, Average Exposure Concentrations (AEC) were closer to intended nominal concentrations: 33.78 ± 2.90 mg l^{-1} in L treatments, and 60.39 ± 7.83 mg l^{-1} in H treatment. AEC were calculated along the experiment for each treatment as the average of the weekly measurement of nitrate.

Biological endpoints

Statistical analysis with GLM detected differences in phytoplankton abundance between controls and treatments (Table I). During the D21 and D28 experimental weeks, phytoplankton abundance in control was higher than in treatments (Figure 1). However, at the end of the experiment both treatments showed higher abundance values than control (Figure 1). Considering the phytoplankton size classes, statistically significant differences were found from day 21 to 48 due to an increase of small size cells (pico-, ulta-, nano- and microzooplankton). However, those differences were not strong enough to discrimitate between controls and treatments in the PCA (Figure 2). In fact, PCA axis 1 and 2 explained only 12% and 3% of the variance respectively being unable to discrimate phytoplankton community structurals changes as a result of the treatments. Moreover, no statistically significant differences in chlorophyll-*a* concentrations were found (Table I).

Table I. Results of the GLM analysis showing the effects of the nitrate treatment on phytoplankton abundance along the experiment. Bold values indicate significant differences ($p < 0.05$). n.a. stand for no available

Endpoint	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D42**	**D49**
Total phytoplankton (cell l^{-1})	0.525	0.691	0.828	**0.017**	**0.008**	0.150	0.070	**0.043**
Picophytoplankton (cell l^{-1})	0.281	0.528	0.745	**0.009**	**0.023**	0.194	0.215	0.398
Ultraphytoplankton (cell l^{-1})	0.187	0.296	0.279	0.111	**0.008**	0.110	**0.028**	**0.015**
Nanophytoplankton (cell l^{-1})	0.122	0.488	0.359	0.644	0.338	0.734	**0.043**	0.200
Microphytoplankton (cell l^{-1})	0.387	0.205	0.184	0.950	0.822	0.480	0.271	0.129
Chl *a* ($\mu g\ l^{-1}$)	0.588	0.528	0.409	0.747	0.390	0.403	0.318	0.373
Oxygen production (mg O_2 hr)	n.a.	**0.035**	0.204	0.129	0.442	n.a.	**0.004**	**0.005**

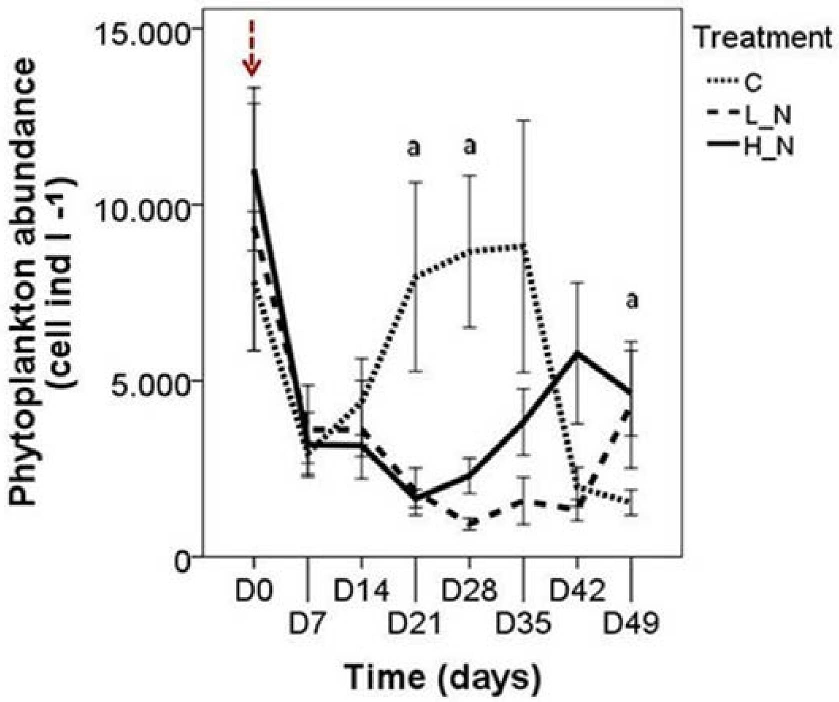

Figure 1. Phytoplankton abundance dynamics (cells l^{-1}) along the experiment. The arrow on D0 indicates the nitrate addition. a indicates statistically significant differences between controls *vs.* treatments.

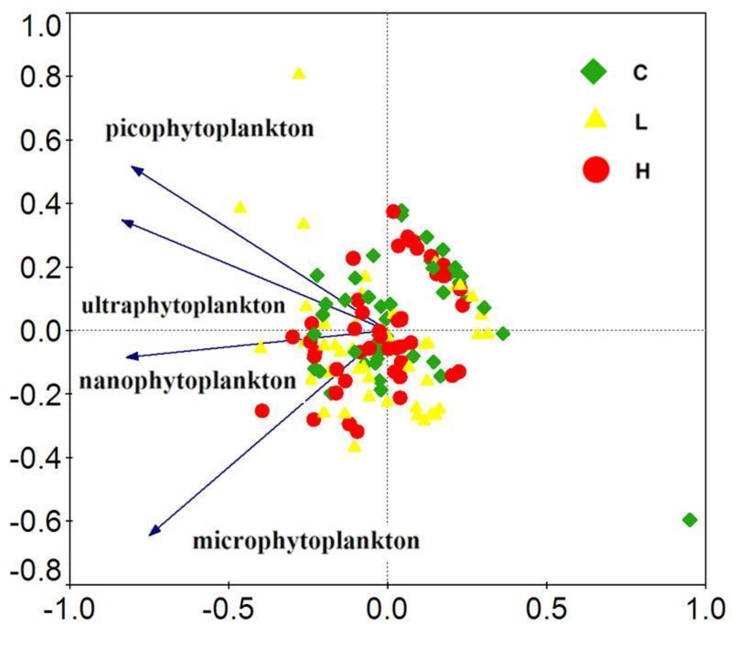

Figure 2. Phytoplankton cell size classes PCA ordination graph along the experiment. Arrows represent the lineal combination of phytoplankton variables with the first and second axes. C, L and H stand for control, low and high nitrate treatments respectively.

The zooplankton community was composed by rotifers, cladocerans, copepods and ostracods. The average of total zooplankton per treatment at the end of the experiment decreased: 132 ± 97 ind l^{-1} in C, 97 ± 30 ind l^{-1} in L treatment, and 62 ± 13 ind l^{-1} in H treatment what represents 145 % in C, 67 % in L treatment and 48 % in H treatments respect to initial zooplankton abundances on D0 (Figure 3). Statistical analysis with GLM detect differences in total abundance and individual abundance of some taxa in specific sampling days (Table II). These results are consistent with PRC (Figure 4), being significant ($p = 0.002$) and denoting differences between controls and treatmens at both abundance and community structure (PRC). In addition, Shannon-Wiener diversity index (1.2 ± 0.61, 1.1 ± 0.20 and 0.8 ± 0.03 bits in the C, L and H respectively) and TPL richness (5 ± 1.14, 4 ± 0.71 and 5 ± 0.89 in the C, L and H respectively) at the end of the experiment were lower in the treatments than in the controls. The TPL less abundance that even dissapear in some weeks in the controls were the cladocera *Macrothrix hirsuticornis*, ostracoda and rotifera; in the Low and High treatment were all taxa of cladocera (*Ceriodaphnia reticulata*, *Alona* sp., *Macrothrix hirsuticornis*) and ostracoda.

Table II. Results of the GLM analysis showing the effects of the nitrate treatment on zooplankton abundance along the experiment. Bold values indicate significant differences ($p < 0.05$). Statistically significant differences when $p < 0.05$. n.p.: no present

Endpoint	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D42**	**D49**
Total zooplankton (ind l^{-1})	0.106	0.272	0.600	**0.008**	0.627	0.625	0.613	0.391
Rotifera (ind l^{-1})	0.454	0.698	0.323	0.667	0.193	**0.022**	0.143	**0.050**
Cyclopoida copepoda (ind l^{-1})	0.131	0.805	0.874	0.305	0.553	**0.039**	0.969	0.440
Calanoida copepoda (ind l^{-1})	0.124	0.439	0.377	0.164	0.384	0.191	**0.033**	0.721
Nauplii (ind l^{-1})	0.246	0.724	0.783	0.026	0.593	0.969	0.816	0.377
Ceriodaphnia (ind l^{-1})	0.210	0.445	0.431	0.085	0.505	**0.000**	**0.000**	**0.000**
Macrothrix (ind l^{-1})	n.p.	n.p.	n.p.	0.605	0.512	**0.081**	**0.000**	n.p.
Alona (ind l^{-1})	n.p.	n.p.	n.p.	0.583	0.193	n.p..	**0.066**	0.863
Ostracoda (ind l^{-1})	**0.021**	n.p.	0.100	n.p.	n.p.	n.p.	0.848	n.p.

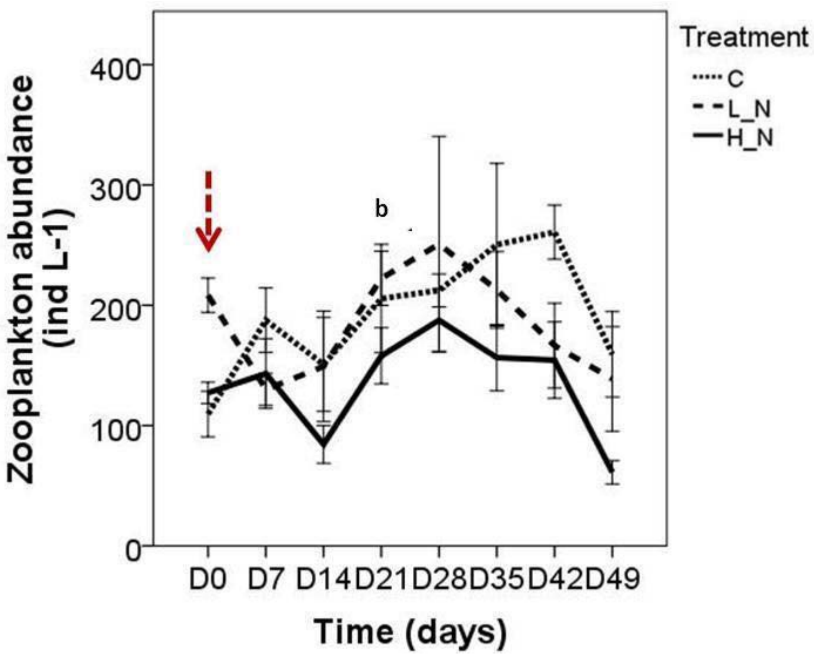

Figure 3. Zooplankton abundance dynamics (cells l^{-1}) along the experiment. The arrow on D0 indicates the nitrate addition. b indicates statistically significant differences between L treatment *vs.*C and H treatment.

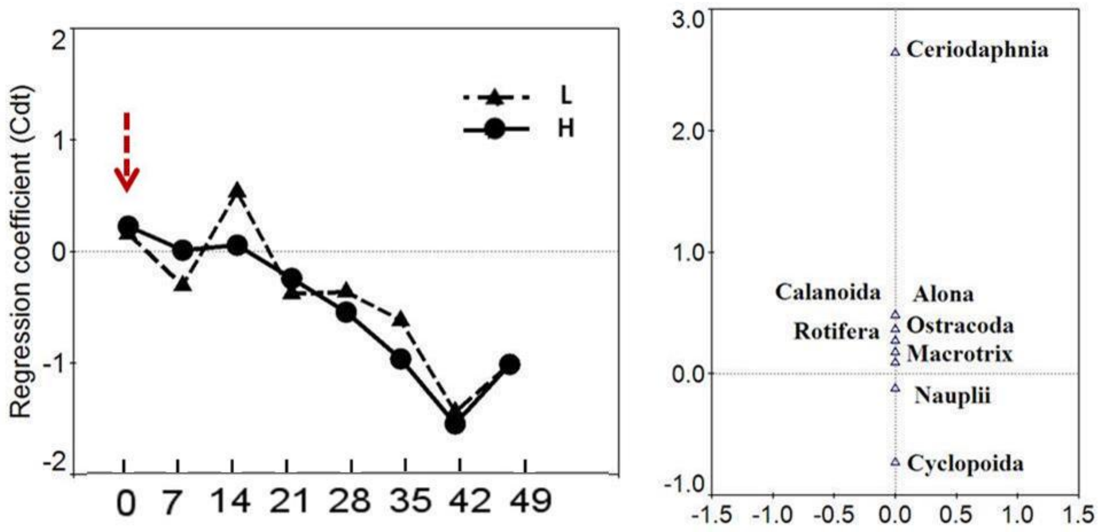

Figure 4. Principal Response Curve (PRC), on the right, ordination method represents the main community respond to the treatment effect over time respect to the controls (dotted line), L treatment (triangles and dotted lines) and H treatment (circles and continuous line). The graph summarizes the zooplankton community respond based on its structure. On the left, the 1-D plot represents the species weights what express the level of affinity that each taxa have with the main trend of the PRC. The arrow on D0 indicates the nitrate addition.

Looking at functional indicators, oxygen production shows significant differences along the experiment but only between the treatments (Table I). Finally, leaf litter decomposition did not show statistical significant differences between control and treatments at the end of the experimental period (p = 0.465).

DISCUSSION

The experimental set up aimed to expose microcosms to the same environmental variability with respect to outdoor environmental condition. It was achieved since there were not statistical significant differences in physical-chemical variables before nitrate addition, consequently the nitrate treatments were the only discriminating factor.

The development of a similar plankton community in all microcosms after the estabilization period was also a critical factor to ensure that nitrate treatments were the only discriminating cause. Total abundance of plankton (both phyto- and zooplankton) on D0 (Figure 1 and Figure 2) did not present statistically significant differences (Table I and Table II) between control and treatments and neither between treatments. Abundance of TPL of zooplankton presented statistically significant differences only in one cathegory (Table II): ostracoda abundance was very low or even not detected in some microcosms what suggest that the differences responde to low detectability factors more than to relevant differences in community structure. Phytoplankton cell size classes did not show statistically significant differences on D0 before the treatments addition (Table I). Therefore, it corroborated initial comparable communities which later differences responded only to treatment effects on the community.

Phytoplankton developed from initial inoculations what suggest no nutrients limitations. Therefore, a higher phytoplankton abundance was expected in the treatments owing to nitrate addition due to a direct effect of nutrients addition, and to an indirect effect through a decrease of grazing presure due to a potential negative effect of nitrate on zooplankton abundance (Ortega et al., 2006; Miracle et al., 2007). Surprisingly, this situation occurred just at the end of the experiment, while phytoplankton abundance was lower in the treatments than in the controls during most of the experimental period (Figure 1). Knight and Notestein (2007) have previously reported a negative relationship between nitrate concentrations and primary production and phytosynthetic activity. Their explanation is a community change from algae adapted to low to moderate nitrate concentrations to submersed aquatic plants as benthic and filamentous algae characterized by higher rates of net productivity but lower gross productivity.

Such phytoplankton community change could explain the lower disolved oxygen in the treatments respect to the controls. Therefore, nitrates effect on phytoplankton may have impact on plankton algae causing its decrease, and favoring the increase of benthic ones. However, nitrate impact did not have effect on the community structure based on both cell size classes of plankton algae measured by citometry and chlorophyll-a. Temporal statistically significant differences of size cell class (Table I) may suggest puntual phytoplankton community changes. Such changes could have been short but intense and missed by weekly sampling routine because phytoplankton life cycle is faster than in zooplankton. In the same way, Chl a relevant changes could have been undetected by weekly sampling. Therefore, weekely sampling could have missed those temporal phytoplankton community changes what could have influenced the zooplankton community change. Flow citometry is considered a convenient tool in ecotoxicological studies (see for example Adler et al., 2007; Jamers et al., 2009) because allows a rapid measurement of a high number of cell features (Olson et al., 1993; Prado et al., 2012) what could be use to detect early signal of toxicant effects. However, our lack of results draw special attention on the importance of life cycles to design an appropriate sampling planning. In the same way, PCA and chlorophyll-*a* concentrations did not present statistical significant differences between control and treatments, therefore failing to discrimate phytoplankton community structurals changes as a consequence of the treatments.

Zooplankton abundance in the treatments only present transient statistically significant differences respect to the controls on D21 (Table). Surprisingly, zooplankton abundance do not decrease as a result of phytoplankton decrease after treatment addtion what would have been interpreted as food limitation. For instance, Kasai and Hanazato

(1995) report the linked response of phytoplankton availability and zooplankton abundance changes. They studied the effect of herbicides on freshwater plankton communities and reported that zooplankton densities decrease as an indirect effect of phytoplankton decrease owing to direct toxic effects of the herbicides. Instead of a decrease of total zooplankton, PRC denotes a zooplankton community change after treatment addition compared to controls: cladocera-dominated communities in the controls changed to copepoda-dominated in the treatments. Community shifts based on an order of sensitivity that increase from copepoda to cladocera under insecticide exposured have been reported previously by Relyea and Hoverman (2006). To the author knowlegde there is not such a consensus about the nutrients effect on zooplankton community shifts. It could be because nutrients most likely have an effect on zooplankton communities owing to indirect effects of phytoplankton changes and not to direct effects as insecticides do. The 1-D plot of the PRC showed that *Ceriodaphnia reticulata* and cyclopoida copepods are the two TPL responding to the treatments. Therefore, L and H treatments are characterized for being richer in copepoda (cyclopoids) versus controls being richer in cladocera (*Ceriodaphnia reticulata*). This specific taxa community change could be the explanation of the no correlation on the total zooplankton and phytoplankton response as would have been expected. And, could indicate an indirect effect of phytoplankton community change from plankton algae to benthic algae impacting zooplankton communty structure. This resuls is in accordance with Hillis et al. (2007) that reported effects of nomensin (antibiotic) on zooplankton community structure and species richness as an indirect result on algae community changes. Nitrate treatment have a direct negative impact in phytoplankton abundance, however zooplankton abundance in the treatments do not decrease owing to a compensatory increase of cyclopoida copepoda. The increase of cyclopoids is most likely and indirect effect of: a) a release of competition from more specialized filter-feeder plankton groups (as *Ceriodaphnia reticulata* more present in the controls than in the treatments) being most likely affected by the decrease of phytoplankton as a result of the treatments impact; and, b) a wider food resources spectrum, cyclopoid copepods are mainly carnivorous of small microcrustacea (i.e. rotifers and nauplii) and also feed on organic and inorganic particles (Holyńska et al., 2003). An increase of cyclopoid copepods over calanoid copepods and cladocerans has been proposed as a bioindicator of trophy status of lakes (Gannon and Stemberger, 1978; Caramujo and Boavida, 1999; Parra et al., 2009). It hightlights, the importance of community structure changes as a potential bioindicator and to understand indirect effect that abundance endpoints may overlook.

Nitrate addition altered zooplankton community structure based on Shannon-Wiener index and richness. Both values were lower in the controls compared to treatment at the end of the experiment. Diversity community changes can provoke drastic and long term effect at ecosystem levels. For instance, Miracle et al. (2007) established a relationship between nutrient addition and a shift to a turbid water state mediated by a zooplankton community change as a result of nutrient disrupting effect on the community. It is critical characterize the community shift because the magnitude of the effects is highly dependent on which species are changing (Cardinale et al., 2006).

Oxygen production as a proxy of production did not detect nitrates effects in this experiment. However, it has been an usefull indicator of community response to disturbances in other studies (Lind, 1979; Cole and Pace, 2000; Downing and Leibold, 2010; Del Arco et al., 2014). For instance, Downing and Leibold (2010) explored how species richness facilitate ecosystem resilience in aquatic food webs under toxic pressures using productivity as one of the studied indicators; and productivity decreased across treatments noticing a negative impact of the toxicants.

Litter decomposition aimed at obtain information of microbial activity through litter decomposition rates. No statistical differences were found by the end of the experiment. However, a shortcoming of the use of litter decomposition in this experiment could be related to a short incubation time for the kind of community under study or low sampling frecuency limited only at the end of the experiment, risking to miss variation in the decomposition rates along the experiment owing to the treatments. Pestana et al. (2009) have used leaf litter degradation as an ecotoxicological endpoint in an outdoor stream mesocosms experiment. The study aimed at assess structural and functional responses of benthic invertebrates and results showed that decompostion was a sensitive endpoint to detect pesticide contamination.

In summary, the experiment aimed at determine if nitrate concentrations within legal limits have negative effect: a) at current nitrate maximun acceptable legal limits (50 mg l^{-1}); and b) at even lower limits (25 mg l^{-1}). It has been found that phytoplankton abundance and zooplankton community endpoints are adversaly affected by both nitrate concencentrations under legal limits. It is in accordance with previous studies as Camargo et al. (2005) that had proposed a 2 mg l^{-1} of nitrate as an appropiate limit for being within the range of more common nitrate concentrations routenily detected. In fact some countries have already lowered legal nitate limits, for instance United Kingdom (18 – 42 mg l^{-1}) and Austria (45 mg l^{-1}).

CONCLUSIONS

Negative effects found at plankton biological endpoints raises the question of how appropiate current legal limits are to prevent environmental risks. These results are consistent with previous studies cited in the discussion claiming the need to lower nitrate concentration in aquatic systems. The authors support the consideration of the precautionary principle and would recommend lowering the nitrate legal limits as have already been done in other European countries. In this sense, more science-based decision should be make. Therefore, more ecological and exposure (mixtures and pulse frequency) realistic chemical assessments are needed.

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CHAPTER 4

"Effects of environmental relevant agrochemical mixtures within legal limits on planktonic community"

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Effects of environmental relevant agrochemical mixtures within legal limits on planktonic community

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ABSTRACT

Freshwater ecosystems face multiple stressors human-induced as mixtures of agrochemicals. Chemicals do not occur alone in the environment. However, most ecotoxicological assessments have studied the effect of single chemicals on aquatic communities. Therefore, more environmental realistic scenarios should be considered. The present study explores how natural assemblages of plankton community respond to environmental relevant agrochemical mixtures within European legal limits. Specifically, the experiment assesses how plankton communities respond to a pulse of a single and mixture pulse of copper sulfate and ammonium nitrate which copper and nitrate concentrations fulfil legal limits. Twenty-five microcosms were used to assess the effects of 4 treatments (n = 5): 1) Nitrate Low treatment (L) of 25 mg l^{-1}, 2) Nitrate High treatment (H) of 50 mg l^{-1}, 3) Copper treatment (Cu) of 0.04 mg l^{-1} of copper, 4) Interaction treatment (I) of 50 mg l^{-1} of nitrate applied together with 0.04 mg l-1 of copper, and the controls (C). Plankton abundance, community structure, diversity and richness were used as structural endpoints; and, oxygen production and litter decomposition as functional indicators. Results show that plankton abundance and community structure are transient but adversely affected by both direct and indirect effects of agrochemical mixtures under legal limits. Concretely, interaction treatment (I) revels how a nutrient enhancement from ammonium nitrate addition counterbalance the toxic effect of copper sulfate most likely as a results of higher phytoplankton availability positively influencing the survival of zooplankters. It highlights the importance of study agrochemical mixtures effects to better understand community responses.

Keywords: agrochemical, mixtures, legal limits, natural assemblages, plankton

INTRODUCTION

Intensive agricultural practices are characterized by multiple applications of agrochemicals causing adverse effects on freshwater ecosystems through run-off events where mixtures occurs (Parra *et al*., 2005; García-Muñoz *et al*., 2010; Stendera *et al*., 2012). Microcosms experiments of single chemicals are commonly used to study toxicant effects in aquatic organisms. Despite of the important information of these experiments, they lack environmental realistic exposure scenarios because they disregard chemicals mixtures in the water bodies. It is well known that chemicals can interact resulting in additive (sum of the individual effects), antagonistic (less than additive) and synergistic (more than additive) effects (Lydy *et al.* 2004; Jonker *et al.*, 2005). Therefore, there is a claim for the need for more assessments considering mixtures of chemicals to better understand direct and indirect effect of agrochemicals inputs on aquatic communities (Laskowski *et al*,. 2010; LeBlanc *et al.*, 2012).

Consumption of manufactured fertilizers based on nitrogen in Europe is translate into 1579 kg ha^{-1} of nutrients; what is much higher than the use of others fertilizers based on phosphorus (139 kg ha^{-1}) and potassium (317kg ha^{-1}) (EUROSTAT, 2009). At the same time, fungicides accounts for 51% of Plan Protection Products (PPP) used in Europe (EUROSTAT, 2007). Therefore, it seems realistic that fertilizers and fungicides co-occur in the environment. Hence, ammonium nitrate as fertilizer and copper sulfate as fungicide have been the selected agrochemicals for the experiment here presented.

Ammonium nitrate is a broad-spectrum fertilizer used worldwide, for instance in crops, rape, beets or pastures. Ammonium quickly transform into nitrates by nitrification processes. Even though nitrates (NO_3^-) naturally occur in aquatic systems, its natural concentrations have drastically increased owing to agricultural run-offs (Camargo *et al.*, 2005). A major consequence of nutrients increase is the eutrophication of aquatic ecosystems, what triggers phytoplankton blooms due to direct (nutrients availability) or indirect effects (zooplankton grazing pressures disruption) leading to water quality degradation (Miracle *et al.,* 2007). Water quality degradation is the most social visible consequence of a web of direct and indirect effects across all trophic levels owing to toxicant exposures. For instance, a microcosm experiment assessing the adverse effects of an insecticide (Chlorpyrifos) in plankton communities showed that changes in grazing pressures owing to a decrease of microcrustacean populations resulted in eutrophication signs (increases of chlorophyll-*a*, algae abundances, dissolve oxygen and pH) what will have adverse effect on water quality parameters(Van Wijngaarden *et al.* 2005). In addition, nitrates effect on non- target aquatic organisms as invertebrate and amphibian have been previously reported (Camargo and Ward, 1995; García-Muñoz *et al*,. 2011).

Copper sulfate is used as fungicide, herbicide and algaecide worldwide (Kungolos *et al.,* 2009), therefore, it can reach aquatic systems both by direct application or run-offs. In addition to the negative effect on environmental values, copper is a public concern as a heavy metal because of its impact on human health (Duruibe *et al.,* 2007). Effects of copper on aquatic systems have been previously reported. For instance, Parra *et al.* (2005) found adverse effect on hatching rates and nauplii survival in copepod (*Arctodiaptomus salinus*) under copper exposures lower than expected field concentrations. Gama-Flores *et al.* (2007) reported a decrease of hatching egg in the rotifer *Brachionus calyciflorus* as low as 16 – 41 % depending on copper exposure concentration and experiment duration respect to the controls. In the same way, there is bibliographic data on the negative effects on amphibian communities (García-Muñoz *et al.,* 2010; 2011). And, there is also data at community levels in aquatic systems (Hedtke, 1984; Havens, 1994; Mastin and Rodgers, 2000; Del Arco *et al.*, 2014).

In the best case scenario, agrochemicals concentrations leaking into aquatic systems would be under legal limits if agricultural good practices are implemented. Nevertheless, legal limits are established base on single test species considering only single chemical exposure and standard species. Even though safety factors are applied to counterbalance limitations of single species test, it may not be enough to prevent environmental risks. In this sense there is a lack of studies under routinely found chemicals concentrations (LeBlanc *et al*,. 2012) as concentrations within legal limits would be; what brings up the question if current legal limits are safe enough.

Therefore, the aim of our study was to evaluate how agrochemicals mixtures pulses within European legal limits affect freshwater ecosystems focusing on the plankton community. The experiment seeks to detect both direct and indirect effect of the agrochemicals mixture and test the following hypothesis: a pulse of mixtures of agrochemical under current legal limits will have adverse effects on the plankton community.

MATERIALS AND METHODS

Microcosms set up

Agrochemical mixtures effects on plankton community was assessed using microcosms with naturally occurring plankton communities from a local pond [Casillas wetland, UTM 30SVG1084 with a surface area of 2.2 ha. (Ortega *et al*,. 2003)]. Microcosms (50 l volume) were filled with 45 l of mineralized water and located outdoor in an experimental wetland infrastructure at the University of Jaén. Vertical hauls with a plankton net (53 µm) were done in the local pond (Casillas wetland) to collect plankton samples which were homogenized and equally distributed among the microcosms. At the same time, superficial sediment layers of the pond were extracted to set a 5 cm layer in all microcosms after its homogenization. Phytoplankton community developed from the inoculated water in the microcosms coming together with the zooplankton and sediment samples. A diverse zooplankton community was developed from the plankton samples and/or hatching from the sediment resistant eggs, with the presence of rotifers, cladocerans, copepods andostracods species.

Plankton community and sediment was inoculated on March 2013 to allow its stabilization and development before the start of the experiment a month later on April 2013. The experiment lasted 49 days (from D0 until D49). The agrochemical mixtures were spiked on a single pulse on D0 after plankton samples were taken to capture initial community conditions. The treatments consisted of four different agrochemical perturbations and the controls (n = 5). The agrochemical perturbations treatments included two pulses of nitrate of 25 mg l^{-1}(low treatment, L) and 50 mg l^{-1} (high treatment, H), one pulse of copper of 0.04 mg l^{-1}(copper treatment, Cu), and a mixture pulse with 50 mg l^{-1} of nitrate applied together with 0.04 mg l^{-1} of copper (interaction treatment, I). The concentrations of nitrate and copper employed in the treatments were selected based on legal limits established by the Council directive 80/778/EEC (revised as Council Directive 98/83/EEC) for nitrates, Council directive 91/676/EEC for nitrates, Boletín Oficial del Estado (BOE, 2011) for copper following the Water Framework Directive (WFD) (Directive 2000/60/CE).

Physical chemical variables

The physical and chemical conditions of microcosms were weekly assessed in the morning (8:00 am). The environmental conditions measured in situ, using a field probe (YSI-556 MPS), were temperature, pH, dissolved oxygen, and conductivity. Alkalinity was measure in the laboratory so then, water samples (100 ml) were taken and transported in cold and darkness conditions for its analysis using a 848 Titrino Plus devise.

Nitrate and copper stock solutions were prepared using ammonium nitrate (NH_4NO_3) and copper sulfate ($CuSO_4$), respectively. A single agrochemical pulse (aliquots of the stock solutions) was spiked on its corresponding treatment on D0 in microcosm's water surface after the first physical-chemical and biological sampling. Microcosms were gently stirred to ensure homogeneous agrochemical distribution on the water column. After the pulse, a sample of 50 ml of water was taken to corroborate if nominal concentrations were reached. A standard laboratory protocol was used to analyze nitrate concentrations (APHA, 1995); ammonia concentrations were measured by a photometric water analysis by a NANOCOLOR KIT (Amonio 3, range from 0.05 – 3.00 mg NH_4^+ l^{-1}); and, copper was analyzed by Inductively Coupled Plasma (ICP) Mass Spectrometry.

Biological endpoints

Phytoplankton community response to the toxicants based on abundance and community size structure endpoint was evaluated by chlorophyll-*a* (Chl-*a*) and flow cytometry measurements respectively. The endpoints were a proxy of phytoplankton abundance (cellular densities measured by cytometry and Chl-*a* concentration) and community size structure (pico-, ultra-, nano- and microphytoplankton cell size classes). Chl-*a* concentration was determined by fluorometry. A calibration curve was calculated based on samples that were filtered through Whatman GF/C glass microfibre filters, and extracted in 90% acetone for 24 h at 4ºC. Cytometry analysis were performed on water samples preserved in glutaraldehide (2%), frozen in liquid nitrogen and stored at 80ºC until the analysis with a BD- LSR Fortessa flow cytometer. Calibration spheres were used to obtain a cell size regression curve: y = 0.008 x –

3.93 ("x" = the mean of the Forward Scatter (FSC), and "y" = cell size of the cells in μm^3). Four cell size populations were studied: picophytoplankton (0.4 – 2 μm^3), ultraphytoplankton (2 - 8 μm^3), nanophytoplankton (8 – 20 μm^3) and microphytoplankton (>20 μm^3). An acquisition time of 180 s at a rate of 60 µl min^{-1} was a set parameter to measure population's cells abundance. These data were analyzed with FACSDIVA software.

Zooplankton community response to the toxicants was assessed weekly by abundance, community structure and diversity based on the lowest taxonomical practical levels (TPL) (Van Wijngaarden *et al,*. 2005; Del Arco *et al.*, 2014). The endpoints assessed were abundance (ind l^{-1}), community structure (PRC), diversity (Shannon-Wiener diversity index) and richness. Zooplankton integrated water samples (0.5 l, plankton net of 60 µm) were weekly taken from each microcosm and preserved in formaldehyde (4% f.c.). The filtered water was returned to the microcosm. Zooplankton was counted, identified and grouped to the following eight taxonomical practical levels (TPL): Ostracoda order, calanoid copepods (*Neolovenulla aullaudi*), cyclopoid copepods (*Acanthocyclops* sp. plus *Metacyclops* sp.), nauplii (calanoida plus cyclopoida), *Ceriodaphnia reticulata*, *Alona* sp., *Macrothrix hirsuticornis* and rotifers order.

Oxygen production was estimated by diurnal oxygen fluctuations as a proxy of ecosystem productivity (Lund, 1979; Cole and Pace, 2000; Downing and Leibold, 2010). It was weekly measured at the start (8:00 am) and at the end (20:00 pm) of the spring-summer photoperiod (12:12) using a field probe (YSI-556 MPS).

Litter decomposition was assessed incubating alder leaves (*Alnus glutinosa*) in order to compare the percentage of Ask Free Dry Mass (AFDM) between controls and treatments at the end of the experiment. One litter bag (10 x10 cm and 1 cm mesh size) for microcosms (initially containing 3 g dry weight of alder leaves) was incubated for the whole experimental period (49 days). After retrieval, litter was rinsed with tap water, oven dried (105ºC for 24h), weighed, ignited (550ºC for 4h) and reweighed to calculate lasting ash free dry mass (AFDM) (Gessner and Chauvet, 1994).

Statistical analysis

Effects of single and mixture pulses of agrochemical in the planktonic community at both structural and functional levels were assess through analysis of variance by General Lineal Models (GLM) followed by a post hoc Tukey test (IBM SPSS statistic 19 software). Prior to GLM analysis plankton data were log (x +1) transformed to meet homoscedasticity and normality assumption. In addition, phytoplankton community structure (pico-, ultra-, nano- and microphytoplankton cell size classes) was studied with a Principal Correspondence Analysis (PCA, CANOCO v4.5 software). Zooplankton community structure was analyzed by Principal Response Curves (PRC, CANOCO v4.5 software). PRC is an ordination analysis based on redundancy analysis ordination (RDA) that allows a graphically observation of the overall community response to the treatments during the experiment compared to the controls (Van den Brink and Ter Braak, 1999; Roessink *et al.*, 2005; Zafar *et al*,. 2012). In addition, the species weights are represented in a complementary graph (1-D plot) which inform about the affinity of the different species with the overall response showed by the PRC. Species can have a positive, negative or null value meaning that the species changes are directly, indirectly or not correlated to the main response trend respectively. Prior to Principal Response Curves, plankton data were ln (ax +1) transformed to counterbalance the influence of taxa no present versus low taxa abundance (Zafar *et al.*, 2012): “a” represent the results of divide 2 by the lowest taxa abundance value of all treatments and “x” the taxa abundance value in each treatment

RESULTS

Physical and chemical variables

All experimental microcosms experienced similar physical and chemical conditions (aside from the agrochemical treatments) as indicated by the lack of any statistically significant differences in temperature ($p = 1$), pH ($p = 0.56$), alkalinity ($p = 0.68$) and percentage of dissolve oxygen ($p = 0.56$). Only conductivity was different in C *versus* H and I treatments on D7 ($p = 0.002$). Respectively, the average measurements during the experiment were 16.6 ± 3.0 ºC; 8.0 ± 0.1 pH;87.3 ± 45.1 bicarbonate mg l^{-1}; 71.7 ± 13.7 % DO and 317.0 ± 77.3 µS/cm.

Nitrate and copper measurements were taken to corroborate intended nominal concentrations and calculated Average Exposure Concentrations (AEC) in each treatment (Table I). During the experiment the average percentage of nitrate concentrations were 51.6 ± 33.9 % (L treatment), 55.3 ± 30.4 % (H treatment) and 47.4 ± 34.5 % (I treatment) of the target nominal concentrations in each treatment. The percentages of nominal copper concentrations were 38.9 ± 11.0 % (Cu treatment) and 46.0 ± 10.5 % (I treatment). Ammoniun levels were lower than 0.05 mg NH_4^+ l^{-1} in all treatments.

Table I. Average Exposure Concentration (AEC) in mg l^{-1} during the treatment period and concentrations measured after nitrate pulse (D0).

Treatments	Toxicant	Nominal Target Concentration (mg l^{-1})	D0 ± S.D.	AEC (mg l^{-1})
L	NO_3^-	25	30.8 ±11.1	14.0 ± 5.5
H	NO_3^-	50	41.0 ± 24.4	24.1 ± 14.4
Cu	Cu	0.04	0.051 ± 0.001	0.015 ± 0.044
I	Cu	0.04	0.058 ± 0.001	0.018 ± 0.042
	NO_3^-	50	46.2 ± 28.9	14.0 ± 5.5

Biological endpoints

Statistical differences in total phytoplankton abundance and cell size classes' abundance between controls and treatments were found in specific sampling days (Table II). Long term consequences were not detected in terms of cell size classes composition of phytoplankton communities based on the absent of discrimination of controls versus treatments in the PCA (Figure 1). The PCA axis 1 explained 63.7% of the variation and PCA axis 2 explained 13.8% of the variation, explaining together 77.5% of the total variance related to the discrimination of treatments base on cell size classes of phytoplankton. However no groups of cell size classes were detected, therefore, PCA shows a main cluster grouping all the microcosms independently of the treatment. Phytoplankton abundance estimated by Chl-*a* showed more permanent statistically significant differences between controls and treatments during three sampling days (Table II, Figure 2).

Table II. Results of the GLM analysis showing the effects of the nitrate treatment on phytoplankton abundance along the experiment. Bold values indicate significant ($p<0.05$) effects. Statistically significant differences when $p < 0.05$. n.m.: not measured

Endpoint	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D42**	**D49**
Total phytoplankton (cell l^{-1})	**0.029**	0.184	0.075	0.134	0.231	0.119	0.391	0.116
Picophytoplankton (cell l^{-1})	**0.009**	0.204	0.095	0.406	0.479	0.224	0.378	0.292
Ultraphytoplankton (cell l^{-1})	**0.077**	0.155	**0.068**	**0.025**	0.278	0.067	0.383	0.185
Nanophytoplankton (cell l^{-1})	0.244	0.161	0.217	0.149	0.071	**0.028**	0.180	0.259
Microphytoplankton (cell l^{-1})	0.117	0.117	0.144	0.317	0.154	**0.048**	0.214	0.357
Chl-*a* ($\mu g\ l^{-1}$)	0.652	0.719	0.262	**0.001**	**0.000**	**0.046**	0.082	0.065
Oxygen production (mg 0_2 h^{-1})	0.107	0.145	0.227	0.406	0.352	0.362	0.272	0.279
Litter decomposition	n.m.							0.244

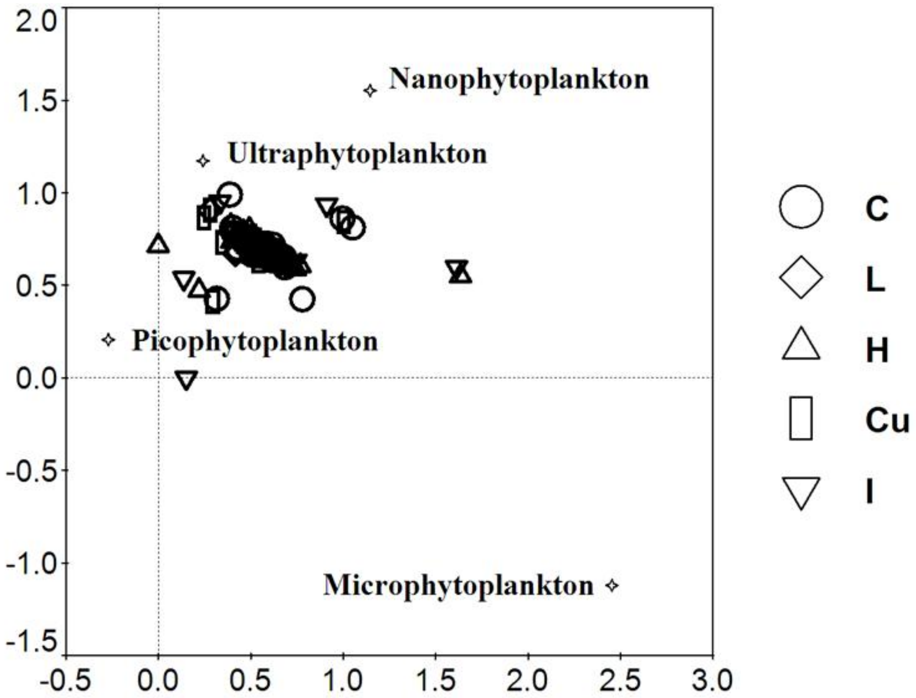

Figure 1. PCA ordination graph. Symbols represent the microcosms ordination by treatment respect to the phytoplankton cell size classes. C, L, H, Cu and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate plus copper respectively.

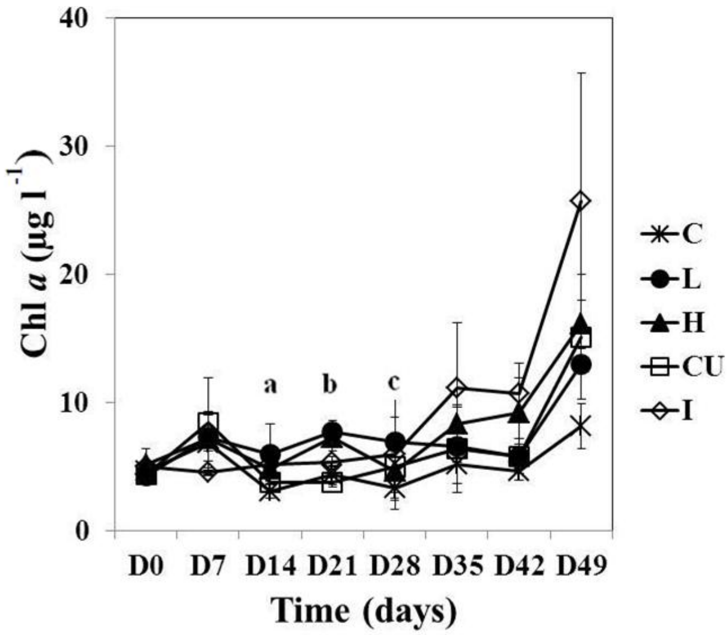

Figure 2. Chlorophyll-*a* mean values in each treatment. C, L, H, Cu and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate plus copper respectively. “a” and “b” denotes

statistically significant differences between controls and L treatments; and, between C and L and I treatments. "c" denotes statistically significant differences between H and Cu treatments.

In relation to zooplankton, the average of total abundance per treatment at the end of the experiment showed two different patterns respect to the controls (251 ± 19 ind l^{-1}): 1) an increase of zooplankton abundance in the treatments with nitrates being 850 ± 1 ind l^{-1}; 583 ± 456 ind l^{-1} and 663 ± 305 ind l^{-1} in L, H and I treatments, respectively; and, 2) a decrease in the Cu treatment, with 177 ± 112 ind l^{-1} (Figure 3). Statistical analysis with GLM detects statistical differences at total abundance and in the abundance of some taxa (Rotifera, Cyclopoida copepods, Calanoida copepods, Nauplii, *Ceriodaphnia reticulate and Macrothrix hirsuticornis*, Table III). PRC results are consistent with the total abundance results and the two community response patterns related to the treatments explained above.

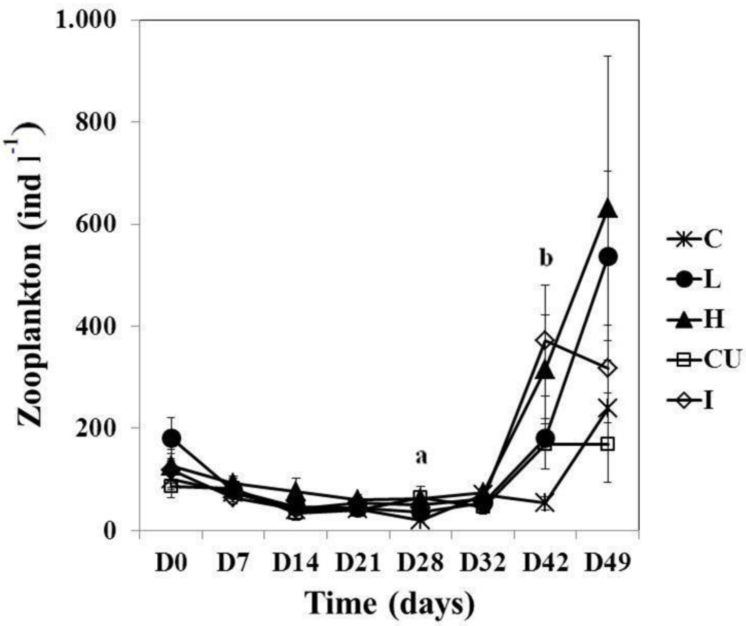

Figure 3. Zooplankton total abundance (ind l^{-1}) values in each treatment. C, L, H, Cu and I stand for controls, low nitrate, high nitrate, copper and interaction of nitrate plus copper respectively. "a" and "b"denotes statistically significant differences between controls *versus* H and I treatments; and, between controls versus L, H and I treatments.

PRC was statistically significant (p = 0.002) and denoted differences between controls and treatments at both abundance and community structure (Figure 3). Shannon-Wiener diversity index (1.69 ± 0.25; 1.32 ± 0.63; 1.27 ± 0.60; 1.22 ± 0.60 and 1.82 ± 0.33 in the C, L, H, Cu and I treatments, respectively) and richness (5.0 ± 0.7; 5.6 ± 0.2; 5.4 ± 0.5; 4.8 ± 0.9 and 5.0 ± 0in the C, L, H, Cu and I treatments, respectively) based on TPL at the end of the experiment did not show statistically significant differences.

Functional indicators, oxygen production and litter decomposition, did not show statistical differences between treatments and controls along the experiment (p > 0.05; Table II).

Table III. Results of the GLM analysis showing the effects of the nitrate treatment on zooplankton abundance along the experiment.Bold valuesindicatesignificant (p<0.05) effects. Statistically significant differences when p < 0.05 n.d.: notdetected

Endpoint	**D0**	**D7**	**D14**	**D21**	**D28**	**D35**	**D42**	**D49**
Total Zooplankton (ind l^{-1})	0.477	0.655	0.131	0.460	**0.009**	0.284	**0.002**	0.135
Rotifera(ind l^{-1})	0.125	0.093	**0.004**	0.094	0.213	0.399	0.153	**0.017**
Cyclopoida copepods(ind l^{-1})	**0.040**	0.560	0.636	0.747	0.793	0.192	**0.012**	0.839
Calanoida copepods(ind l^{-1})	**0.026**	0.148	0.332	0.347	0.733	0.147	0.445	0.069
Nauplii(ind l^{-1})	0.724	**0.057**	0.172	0.565	0.700	0.693	0.155	0.400
Ceriodaphniareticulata(ind l^{-1})	0.630	0.756	0.115	0.163	0.115	0.181	**0.051**	0.078
Macrothrixhirsuticornis (ind l^{-1})	0.101	n.d.	n.d.	n.d.	n.d.	n.d.	**0.171**	0.055
*Alona*sp. (ind l^{-1})	0.103	0.182	n.d.	n.d.	n.d.	0.109	0.445	n.d.
Ostracoda (ind l^{-1})	0.041	0.093	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

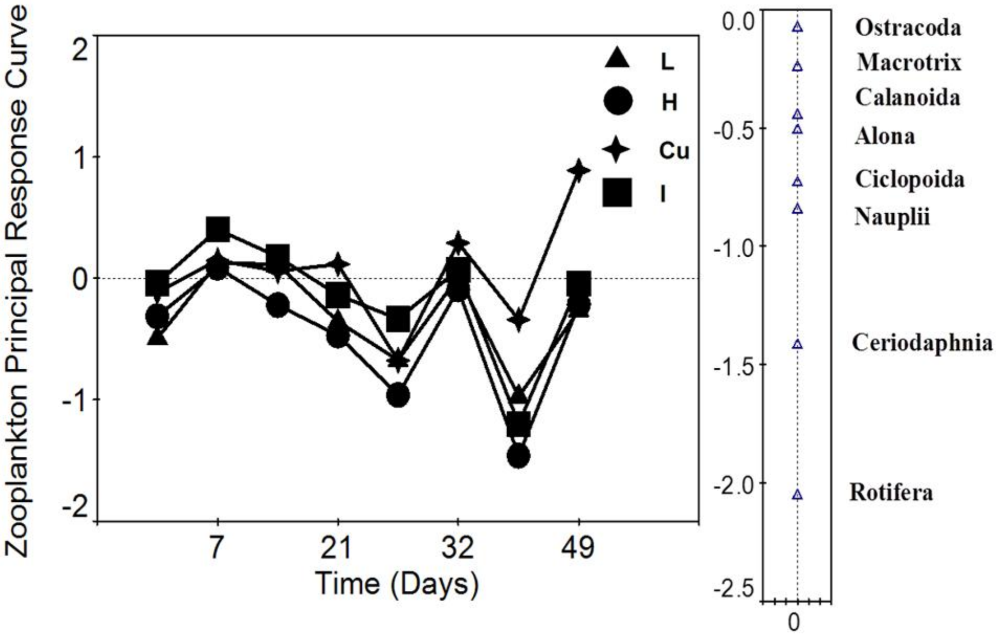

Figure 4. Principal Response Curve (PRC), on the left, ordination method represents the main community respond to the treatment effect over time with respect to the controls (dotted line in the middle of the graph represent the C and the lines with triangles, circles, stars and squares represent L, H, Cu and I treatments). The graph on the right summarizes the zooplankton community response based on its more influent taxa; it represents the species weights expressed as the level of affinity that each taxa had with the main trend of the PRC.

DISCUSSION

The experiment was intended to explore how plankton community of freshwater ecosystems will respond to disturbances caused by mixtures of commonly used agrochemicals within current legal limits. The responses presented in the results will be linked to the experimental treatments because nominal concentrations of individual and mixtures agrochemicals were achieved, and no long lasting physical-chemical differences between microcosms were detected.

Phytoplankton abundance denotes transient effect at individual sampling days between controls and treatments both before (D0) and after the treatments (D7). It could mean that either the phytoplankton community develops differently in the microcosms, which seem unlikely since all the inoculations of plankton, physical-chemical and environmental conditions were the same; or that the sampling procedure was not the most appropriated for the present microcosms experiment. In relation to the second aspect, 4 ml volume samples were taken to run the cytometric analysis. Although the homogenization of the microcosms was done before the sample, it is possible that could have been insufficient to capture phytoplankton community abundance and its response to the treatment. In fact, a more commonly used Chl-*a* measurement was able to detect differences on consecutive sampling days between controls and treatments (Table II, Figure 2). Cytometry has the potential to be a powerful tool in ecotoxicology because it allows a fast measurement of diverse cell features (Adler *et al.,* 2007; Jamers *et al.,* 2009; Prado *et al.,* 2012). However, its use in microcosms experiments aiming at capture phytoplankton community response may require further adjustments to be able to detect large, colonies, phytoplankton. Changes in phytoplankton cell size could be an appropriated fast endpoint to complement the explanation of the changes registered in zooplankton abundance and community shifts in relation to indirect effects. Information about abundance of the diverse cell size classes could inform data about edibility. Edibility of phytoplankton modulates zooplankton grazing capacity (Miracle *et al.,* 2007; Holt, 2008; Scheffer *et al.,* 2008; Cumming *et al.,* 2013) what could influence zooplankton fitness and consequently its response to the toxicants. As mentioned above, the Chl-*a* concentration denoted an effect of treatments on the total abundance of the phytoplankton community, increasing in all treatments. An increase of phytoplankton in Cu treatments may be related to an indirect effect related to a decrease of zooplankton owing to copper toxicity (Parra *et al.,* 2005), or as a result of more nutrients availability favoring phytoplankton growth. On the contrary, a negative effect of Cu treatments on phytoplankton would have been expected based on an adverse effect on algae growth (Koutsaftis and

Aoyama, 2006). Phytoplankton availability seems to have favored the increase of zooplankton, both growing together along the experiment. It could have been expected a decrease of phytoplankton under higher pressures of zooplankton growth. One possible explanation could be that treatments are dominated by rotifers, which grazing capacity would be too low to control phytoplankton development. In this sense, Miracle *et al.* (2007) studied, with mesocosms in a Mediterranean shallow pond, the effect of nutrients on rotifers and reported that their grazing capacity is much lower than macrozooplankton grazing activity. The most interesting effect came from the treatment I, suggesting that nitrate addition seems able to interfere the negative toxic effect of copper. This result could be explained by a compensation of the negative effects of copper as a consequence of major food availability due to nutrients addition favoring the phytoplankton growth. This modulation capacity does not mean the absence of negative effect respect to the controls, but it point out that the community response is highly more complex than expected under mixture of chemicals because the occurrence of indirect effects. This result is especially relevant because highlights the indirect effects as a result of agrochemical mixtures with highly diverse mode of action and targets. In this case, the interaction between a fertilizer and a fungicide show similar phytoplankton responses but mediated by different indirect effects.

Zooplankton abundance denoted differences between controls and treatments. The total abundance of zooplankton (ind l^{-1}) shows a zooplankton response by the end of the experiment with two different patterns. Total zooplankton abundance increase in the treatments with nitrates (L and H) even in the treatment I, where nitrates are added together with copper. By contrast, total zooplankton abundance decrease in the Cu treatment. It could indicate an indirect effect related to an influence on the individuals response to copper toxicity owing to higher food availability resulting in better individual fitness. Caramujo and

Boavida (1999) stated the importance of food quality for reproductive cycles and development stages of zooplankton taxa. In addition, Gui and Grant (2008) studied the influence of food availability combined with toxicants exposures on *Drosophyla melanogaster* and reported that specific food availability could modulate toxicant exposures under specific population densities and toxicant exposures. In accordance with this premise, Chl-*a* increases specially in the treatments with nitrate (L, H and I) in the last sampling days respect to the controls and the copper treatment (Cu). PRC also denotes two different response patterns in terms of community structure by the last sampling days. Treatments with nitrates (L, H and I) are similar to the controls being richer in rotifers by the end of the experiment; while Cu treatments differ from the controls being poorer in rotifers. Therefore, the PRC results support the hypothesis of an indirect effect related to phytoplankton abundance as previously discussed. Rotifers within this community could be the most sensitive individuals to copper. Therefore rotifers population decrease under Cu treatments; while, the decrease is softer in the I treatment owing to the addition of nitrate that could lead to an increase of phytoplankton resulting in more food availability for rotifers that compensate the toxic effect of copper. Copper exposure were 0.04 mg l^{-1} what is within legal limits, therefore, no effect on rotifers would have been previewed. In fact, other studies have reported acute test (48-h exposure) of neonate's rotifer species (*Lecane hamata* and *L. quadridentata)* resulting in LC_{50} values of 0.06–0.33 mg l^{-1} (Pérez-Legaspi and Rico-Martínez, 2001). However, other study has described a lower value of LC_{50} (24-h exposure) for *Brachionus calyciflorus* of 0.02 mg l^{-1} for copper sulfate (Snell and Persoone, 1989). Other potential sensitive taxon was *Ceriodaphnia reticulata* as the PRC denoted what is partially supported by the published LC_{50} (48-h exposure) of 0.003 mg l^{-1} for copper sulfate for other congeneric specie, *Ceriodaphnia dubia* (Suedel *et al.*, 1996). Nevertheless, *Ceriodaphnia reticulata* did not show a clear response to the treatments in this experiment. Broadly there is an order of sensitivity increasing from copepods to cladocerans in the literature (Hanazato 1998). Nonetheless, in our experiment copepods also do not respond to the treatments, possibly because they present an LC_{50} higher (Lalande and Pinel-Alloul, 1989) than the copper concentration tested in this study. Consequently, rotifers could be the most sensitive taxa within this specific community because they showed the clearest response to the treatments. Additionally, the most relevant fact is that the mixture of agrochemical may modulate rotifers response. One interesting aspect is the possible influence of this result on the trophic web. These effects are known as bottom-up effects and suggest that small alterations in the base of the trophic web, that are usually ignored, may mean a community shift or processes rates changes, leading to impacts of higher magnitude (Scheffer *et al.,* 2008). In this respect, Hanazato (1998) and Zagarese (1991) described the consequences of zooplankton abundance and taxa shifts on the whole community structure influencing spring clear-water phase in lakes and fish larvae development.

Shannon-Wiener diversity index, TPL richness as structural endpoints, and, oxygen production and litter decomposition, as functional endpoints did not detect any statistical significant difference. This is not unforeseen, no effect or transient effect of some endpoints were expected since the experiment was done using agrochemical concentrations under legal limits. However, those slight effects reflected by zooplankton (abundance and PRC) and phytoplankton (Chl-*a*) were indeed hypothesized. That is, current legal limits are based on single species test and even if security factors are applied the root of the studies may lack complexity to capture community responses (Van den Brink, 2013; De Laender and Janssen, 2013). Therefore, that was the reason why even exposures within legal limits were hypothesized to have negative adverse effects.

In conclusion, results show that phytoplankton abundance, zooplankton abundance and zooplankton community structure are adversely affected by both direct and indirect effects of agrochemical mixtures under legal limits. It could be argued that recovery capacity could overcome such transient impacts; however, under a precautionary principle such adverse effects in assumed protected communities raise concerns about long-term impacts.

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CHAPTER 5

"Freshwater plankton food web responses to pulses of agrochemical mixtures"

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Freshwater plankton food web responses to pulses of agrochemical mixtures

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ABSTRACT

Intensive agriculture is a leading perturbation of aquatic systems compromising biodiversity and ecosystem services. Predicting how freshwater systems will respond to complex agrochemical disturbances is difficult because of their repeated nature, the potential for chemicals interactions, and for both direct and indirect effects. This study explores how zooplankton communities respond to temporally repeated pulses of nutrients and insecticides, singly and in combination, in order to understand how agrochemical mixtures affect aquatic system integrity and biodiversity. We conducted an experiment to assess the ecotoxicological effects of a commonly used insecticide (chlorpyrifos) and nutrients (nitrogen and phosphorus) on plankton communities. Microcosms (300 L) were established outdoor for 10 weeks. The treatments consisted of controls and four different perturbations (insecticide, nutrients, insecticide and nutrients applied synchronously applied, and insecticide and nutrients asynchronously applied), each with 5 replicates. Zooplankton abundances, community structure and biodiversity were used as structural indicators. Chlorophyll *a* and ecosystem productivity were used as functional indicators. We found significant differences between treatments and controls at structural levels. Principle response curves of zooplankton communities showed a shift in composition towards copepod dominated communities in treatments that received insecticide. We did not find evidence for additive or synergistic effects of insecticides pulses and nutrient pulses together. In conclusion, the insecticide disturbance had strong direct and indirect effects on zooplankton community response but nutrient pulses alone and in combination with insecticides had little visible impact on aquatic communities.

Key words: agrochemicals; mixtures; frequency; assessment; risk; plankton

INTRODUCTION

Anthropic pressures are affecting ecosystems resulting in biodiversity losses worldwide, and this fact is especially dramatic in freshwater ecosystems (Sala et al., 2000). One of the most significant pressures facing the ecological integrity of freshwater ecosystems is intensive agriculture characterized by applications of pesticides and fertilizers (Parra et al., 2005; Stendera et al., 2012). As a result of repeated agrochemical application and run-off events, freshwater ecosystems often receive pulses of agrochemical mixtures at varying frequencies with consequences for aquatic ecosystem biodiversity, structure and function (Troncoso et al., 2000; Parra et al., 2005). In order to understand the ecological relevance of pulsed toxicant mixtures for freshwater ecosystems, we need to not only understand the effects of single toxicants on single species but we must also evaluate the effects of pulses of toxicant mixtures within a context of complex ecological interactions (De Laender and Janssen, 2013). This approach will also result in more ecological realistic risk assessments (ERA) useful for science-based policy decisions.

Two common agrochemical applications that have particularly strong effects in aquatic ecosystems are fertilizers and insecticides (Bronmark and Hansson, 2002). Fertilizers often enter aquatic ecosystems in repeated pulses, particularly in agricultural areas with diverse crops, complex agrochemical application timing and variable rainfall events (FAO, 2006; LeBlanc et al., 2012; Smith et al., 2001; Haygarth et al., 2012). Fertilizer run-off generates an overall increase in nutrient input into freshwater ecosystems, leading to many changes including enhanced productivity, a reduction in biodiversity (Porter et al., 2013), and altered planktonic structure (Miracle et al., 2007; Hall et al., 2004). When nutrients enter ecosystems as repeated pulses, nutrients can have additional effects on community structure by altering species co-existence mechanisms

(Holt, 2008) and disrupting consumer-resource interactions which, for instance, can allow plants to escape top-down control (Scheffer et al., 2008; Cumming et al., 2013)

Similar to nutrients, insecticides often enter ecosystems in repeated pulses depending on rainfall events and application schedules designed to maximize control of pests and disease (FAO, 2001; Reinert et al., 2002; Hoang et al., 2007; Earl and Witheman, 2009). Insecticides typically cause high mortality in the zooplankton community, especially for larger cladoceran species (Kreutzweiser et al., 2004; Hanazato, 1998; Van den Brink et al., 1996; Downing et al., 2008; Brock et al., 2006; Brausch and Salice, 2011). The reduction in zooplankton and the shift towards smaller zooplankton species such as rotifers and copepods has additional indirect effects on phytoplankton by reducing grazing pressure (Hanazato, 1998). The pulse interval of insecticides is important because it may cause sublethal effects and can allow or prevent recovery of the community between pulses, resulting in pulses that are either independent or cumulative in nature (Hoang et al., 2007; Reinert et al., 2002). Additional work has shown that species and communities respond differently to single versus chronic pulses of contaminants (Van Wijngaarden et al.,2005) and to repeated exposure of a single type versus multiple types of contaminants (Tlili et al., 2011).

In order to improve our understanding of the consequences of agricultural disturbances on aquatic ecosystems, we need to study them as they occur in natural ecosystems. Agricultural disturbances are often complex and involve multiple chemicals that may be pulsed repeatedly, either singly or in various combinations (LeBlanc et al., 2012; Borgert et al., 2004). Despite the complexity of human-induced disturbances in natural ecosystems, most toxicological evaluations have studied the effects of single pulses of a toxic substance on individual species that are well-known bioindicators (Hoang et al., 2007; Earl and Whiteman, 2009; García-Muñoz et al., 2011). Some studies have explored how mixtures of chemicals or disturbances affect taxa, but these studies still typically explore the response of single species rather than entire food webs or communities (Reinert et al., 2002; Hurd et al., 1996; Le Blanc et al., 2012; Brock et al., 2000; Jonker et al., 2005). Chemicals in mixtures often behave differently than predicted based on their actions as single chemicals because chemicals in mixtures often interact and they may have different or similar effects on organisms, making it difficult to determine if the effects of multiple chemicals on organisms will be independent (Deener, 2000; Verbruggen and Van den Brink, 2010), antagonistic or synergistic (Lydy et al., 2004; Le Blanc et al., 2012). In marine systems, multiple stressors were most often synergistic, but varied with the focal level of response (e.g. population level vs community level), trophic level, and the particular combination of stressors (Crain et al., 2008), suggesting a lack of general response of multiple stressors.

The aim of our study was to evaluate how multiple stressors in freshwater systems, in the form of repeated insecticide (chlorpyrifos) and nutrients (nitrogen and phosphorus), affect the integrity and biodiversity of freshwater ecosystems with a particular focus on the zooplankton community. Nutrients and chlorpyrifos, a broad-spectrum organophosphate pesticide extensively used for agricultural purposes worldwide, were chosen because they represent common agrochemical contaminants that are frequently applied either alone or in combination. Agrochemicals are often applied repeatedly over a growing season which, in combination with run-off from sporadic rainfall events, can lead to pulsed and repeated inputs of nutrients and insecticides into aquatic ecosystems (Brock et al., 2000; Sala et al., 2000). Additionally, insecticides like chlorpyrifos are likely to occur both synchronously and asynchronously with nutrients in runoff events (FAO, 2001; FAO, 2006; Reinert et al., 2002). Pulses of chlorpyrifos are predicted to temporarily reduce zooplankton abundance and increase phytoplankton abundance through indirect effects, whereas pulses of nutrients are expected to temporarily increase abundance of phytoplankton and zooplankton may or may not be able to respond to variable amounts of productivity. Both are likely to have a negative effect on zooplankton diversity.

We conducted the study using outdoor aquatic microcosms in order to explore community responses under complex and more realistic toxic scenarios (Shurin, 2001; Hall et al., 2004). Specifically, we seek to understand how the timing and combination of pulses of mixtures of agrochemicals affects planktonic food webs. To do this, we assembled diverse plankton communities collected from nearby natural ponds and then applied repeated pulses of nutrients and insecticides alone and in combination both synchronously and asynchronously. We expected pulses of nutrients and of chlorpyrifos to increase phytoplankton and decrease zooplankton, respectively, due to the known direct effects of each chemical. We further predicted that combinations of nutrient and chlorpyrifos pulses would have even stronger effects on the community due to both direct and indirect effects mediated through the food web, but that the community response may differ if pulses are synchronous versus asynchronous.

METHODS

The response of freshwater planktonic food webs to pulsed disturbances was explored experimentally using replicated pond ecosystems established in microcosms. Microcosms were maintained outdoors at Ohio Wesleyan University's Kraus Nature Preserve, Delaware, OH USA. Twenty-five microcosms were established in plastic tanks of 87.6 cm diameter and 45 cm depth. They were filled with 270 liters of well water and covered with mesh lids to avoid immigration by larger organisms. The water was amended with nutrients to bring the concentrations up to 800 μg N / l and 57 μg P /

l which is the average concentration of local ponds. Previous work has shown that nitrogen and phosphorus are lost in these experimental microcosms at the rate of approximately 5% per day (Downing et al., 2008). In order to maintain these target concentrations all tanks received nutrient inputs over the experimental period to match the loss rate of 5% per day, but the size and frequency of inputs varied with treatment as described in more detail below. Microcosms were established in May 2012 and the experiment concluded in August 2012, with the experiment lasting a total of 10 weeks. Microcosms were first inoculated in early May with a naturally diverse assemblage of phytoplankton collected from 10 local ponds, strained through a 30 µm net to remove large zooplankton and macroinvertebrates. After two weeks, a diverse assemblage of zooplankton was collected from the same 10 ponds using 35 µm plankton net and added to the microcosms after macroinvertebrates were removed. The microcosms were exposed to natural environmental variability with respect to temperature and rainfall.

The experiment consisted of 5 treatments including a control treatment (C) and four pulsed treatments of nutrients and/or insecticides. The pulses of nutrients and insecticides occurred every two weeks and were designed to represent possible scenarios in ecosystems that experience agrochemical inputs. The pulsed treatments included: insecticide pulse every 2 weeks (I), nutrient pulse of nitrogen and phosphorus every two weeks, (N), nutrients and insecticide pulsed simultaneously every two weeks (NI), and nutrients and insecticide pulsed in alternating weeks (N_I). Each treatment had five replicates. Control (C) and insecticide (I) treatments received small and frequent nutrient inputs consisting of additions of 93.3 µg N L^{-1} and 6.65 µg P L^{-1} three times per week for the duration of the experiment to maintain target water column concentrations based on the 5% loss rate known to occur in these experimental systems (Downin et al. 2008). Insecticide (I) pulses were delivered as 2 µg L^{-1} of chlorpyrifos every two weeks, representing an environmentally realistic worst case scenario observed in water bodies given the fact that toxicant concentrations can rise up to several orders of magnitude after rainfall events (EPA, 2006; Poletika Woodburn and Henry, 2002; Rabiet et al., 2010). Nutrient (N) pulses were delivered as 560 µgL-1 of nitrogen and 39.9 ug L^{-1} of phosphorus every two weeks. This treatment regime resulted in microcosms that all had the same average amount of nutrients added over the course of the experiment.

Nutrients were added in the form of Na_2HPO_4 and $NaNO_3$. Nutrients were diluted in water and delivered via pipette in 5ml increments to the microcosms. Analytical grade Chlorpyrifos (Sigma Aldrich, Chlorpyrifos PESTANAL ®) was diluted in acetone. Two mls of the acetone/Chlorpyrifos mixture were added to the insecticide treatments and 2 mls of pure acetone were added to all other microcosms to serve as a control for potential effects of acetone (applied at approximately a 0.0001% concentration). All microcosms including controls were gently stirred immediately after application of nutrients or insecticides to homogenize the concentrations in the water column.

Microcosms were sampled weekly for 10 weeks. Physical-chemical measurements (temperature, pH, % dissolved oxygen and conductivity) were taken using field probes (YSI 550 Oxygen probe, YSI Sonde, Yellow Springs, Ohio, USA). Water samples (300 mL) were taken, cold stored and transported to the laboratory to perform chlorophyll extractions. Zooplankton integrated water samples (16 L) were taken from each tank, filtered through a plankton net of 35 µm, handpicked to remove unwanted particulates (e.g. clumps of detritus, sand, etc.) and preserved in the lab with Lugols solution. The filtered water was returned to the microcosm. Zooplankton were identified to taxonomic practical levels (Ostracoda order, cyclopoid copepods, calanoid copepods, nauplii, copepodite, *Bosmina sp.*, *Scapholebris sp.*, *Chydorus sp.*, *Alona sp.*, *Pleuroxus sp.*, *Simocephalus sp., Ceriodaphnia sp.)* and abundances and Simpson biodiversity indices were estimated. Chlorophyll-*a* (Chl-*a*) concentration was measured via extraction (Welschmeyer 1994) and a flurometer (Turner Designs 700) to estimate the response of phytoplankton (Hedtke 1984). Ecosystem productivity was approximated by diurnal oxygen fluctuations (net productivity as gross productivity minus the respiration of all organisms) (Lund, 1979; Cole and Pace, 2000; Downing and Leibold, 2010).

The response to disturbance of communities and ecosystems was explored by quantifying the degree to which pulsed treatments differed from controls by the end of the experiment. Eight weeks is considered enough time to capture initial signals of recovery in plankton communities due to short generation times and resistant structures such as resting eggs because these features are crucial factors controlling the community recovery (Van den Brink et al., 1996; Brock et al., 2000).

Physical-chemical and biological response variables were compared among microcosms using univariate and multivariate analyses with SPSS 19 software. Univariate ANOVA and a post hoc Tukey test were used to determine the differences between controls and treatments. Zooplankton data were log transformed (log (x +1)) to meet normality assumption. Prior to analysis, data were tested for normality and homoscedasticity. Ordination analysis was performed to test the plankton community compositional responses. A Principal Response Curve (PRC) was done using CANOCO v4.5 software to analyze the zooplankton data set. PRC is a technique based on Redundancy Analysis (RDA) ordination techniques (Van den Brink and ter Braak, 1999). The PRC analysis results in a diagram displaying the principal response of the community (y-axis) for all sampling days (x-axis) by showing the deviations in time of the treatments compared to the controls. The species weights are presented in a different graph (1-D plot) which reveals the affinity of the different species with the principal community response. The species with a high positive weight are the most correlated to the main response showed by the PRC, while the species with a negative weight show the contrary trend to the main one reflected by the PRC. Species with weight close to zero means no response or very dissimilar to the main response. In order to test differences among treatment for each week in the PRC, the following steps were done: first, abundance data of zooplankton was ln (ax +1) transformed in order to avoid confounding effects between low abundance and no presence of the taxa ("x" stands for zooplankton abundance value and "a" is calculated by dividing 2 by the lowest zooplankton abundance value higher than zero); second, PCA was calculated to obtain the first axes values; and third, statistically significant differences per week and treatment were test with Dunnet post hoc test using SPSS 19 software (Van den Brink et al. 1999; Roessink et al. 2005; Zafar et al. 2012).

RESULTS

All experimental microcosms experienced similar physical and chemical conditions throughout the experiment (aside from the chlorpyrifos, N and P) as indicated by the lack of any statistically significant differences in dissolved oxygen, pH, temperature and conductivity. Consequently, the treatments themselves did not introduce additional physical or chemical differences between treatments.

The zooplankton community was predominantly composed of cladocera, copepoda and ostracoda. Nine different taxa were found: *Bosmina sp., Scapholebris sp., Chydorus sp., Simocephalus sp., Alona sp., Ceriodaphnia sp., Pleuroxus sp.,* cyclopoida copepoda, calanoida copepoda and ostracoda. Copepodite and nauplii within the copepods were counted and used for the ordination analysis. Zooplankton abundance was statistically different between the C and I, NI and N_I treatments (ANOVA, F = 10.756, $p < 0.001$, Tukey´s test) (Figure 1).

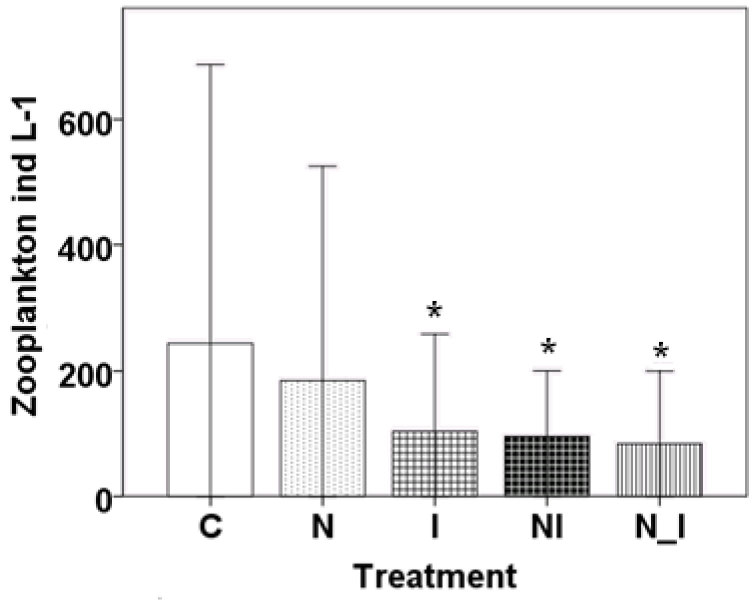

Figure 1. Zooplankton mean (± SD) abundance (ind L^{-1}) in each treatment. * An asterisk denotes statistically significant differences from the control.

Zooplankton composition was also different between treatments as revealed PRC. The PRC was performed to characterize the degree of change and duration of the treatment effects upon the zooplankton community (Figure 2). PRC was significant (F=58.586, p-value = 0.002), indicating that the disturbance treatments had detectable effects on the zooplankton. The variance explained by the first axis was 20.8 %, while the second axis expalined 3.2%. Results of Dunnet post hoc test based on the PRC agree with ANOVA results because statistically significant differences were found every week between C and treatment with insecticide (I, NI and N_I) but not with the N treatment. 1-D plots show the species weights (Figure 2) and indicate how different taxa are correlated to the main community response. 1-D plots show an increase in copepods in I, NI and N_I treatments and a decline in cladocerans and ostracods. Cladocera and ostracoda were more abundant in the controls and N treatments communities. In summary, the two patterns that are most evident are 1) most of the C and N treatments are associated with cladocera-dominated communities, and 2) I, NI and N_I treatments are associated with copepod dominated communities.

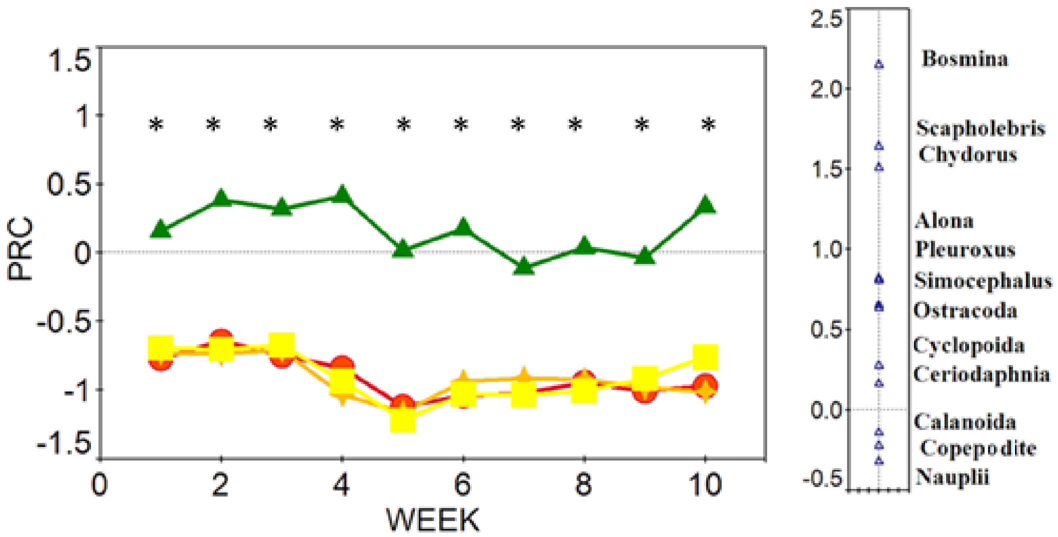

Figure 2. Principal Response Curve (PRC), on the left, ordination method represents the main community respond to the treatment effect over time with respect to the controls (dotted line, triangles, circles, stars and squares represent C, N, I, NI and N_I). The graph on the right summarizes the zooplankton community response based on its taxonomy; the 1-D plot represents the species weights expressed as the level of affinity that each taxa had with the main trend of the PRC. * An asterisk denotes statistically significant differences from the control.

In addition to these compositional shifts, biodiversity (Simpson’s index) decreased significantly in the treatments treated with insecticide (ANOVA, F = 13.718, $p < 0.001$; Figure 3).

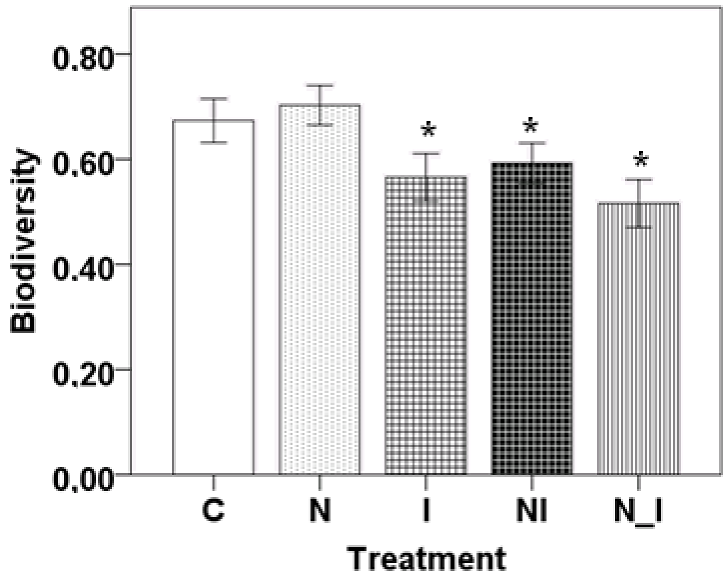

Figure 3. Simpson Biodiversity index mean (± SD) value in each treatment. * An asterisk denotes statistically significant differences from the control.

Chl *a* was statistically different between C and N treatments versus I treatments ($F = 7.446$, $p < 0.001$) (Figure 4), with chlorophyll generally highest in the I, NI and N_I treatments. Productivity average values were between 0.196 mg O_2 per hour in controls and 0.203 mg O_2 per hour in the N treatments. There were no significant differences ($F = 1.452$, $p = 0.218$) between the controls and the treatments (Figure 5).

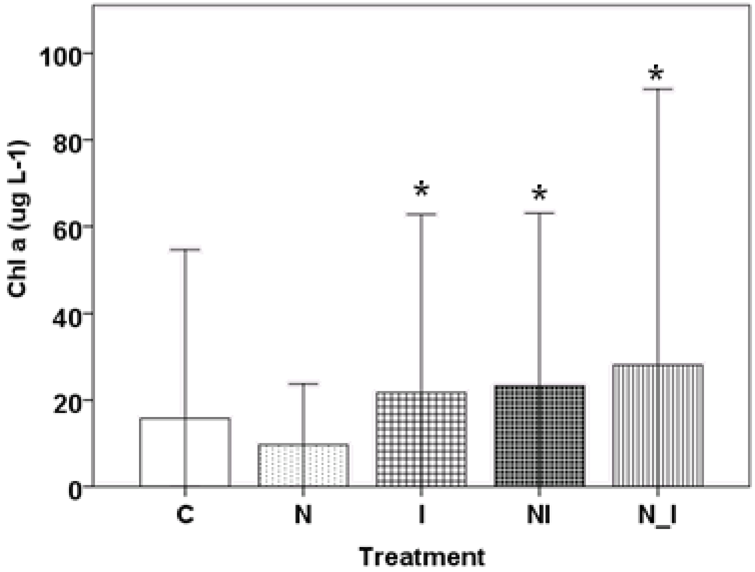

Figure 4. Chlorophyll *a* mean (± SD) values in each treatment. * An asterisk denotes statistically significant differences from the control.

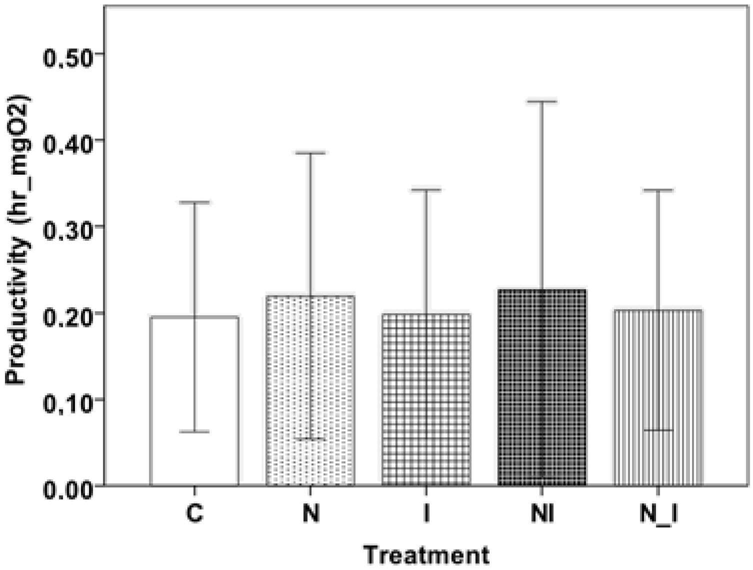

Figure 5. Productivity mean (± SD) values in each treatment.

DISCUSSION

The microcosm experiment was designed to explore how freshwater ecosystems will respond to complex agrochemical disturbances that are often pulsed and in combination in nature. The results obtained show that all measured biological indicators except for ecosystem productivity (expressed as O_2 production) revealed significant responses to pulsed disturbance treatments. First, zooplankton abundance, biodiversity, community composition and Chl *a* showed differences between controls and N treatments versus treatments with insecticide (I, NI and N_I). Second, PRC revealed a community shift from a cladoceran-dominated to a copepod-dominated community in response to the insecticide treatment with corresponding changes observed in the Simpson diversity index. Third, indirect effects of insecticides were detected when analyzing Chl *a* changes; I, NI and N_I resulted in an increase in phytoplankton either by an increase in phytoplankton abundance or a shift in community structure in terms of increasing cells richer in Chl *a* due to a decrease in grazing pressure related to the negative effects of the insecticide on zooplankton populations (Figure 4). And fourth, the effects of insecticide on the response of organisms in wetland communities were drastic and they were not modulated by either its asynchronous or synchronous mixture with nutrient pulses. In summary, our hypothesis was partially supported since agrochemicals had direct and indirect effects on the community, and the community responded differently to insecticide and nutrient pulses. However agrochemical mixtures did not have a stronger impact than pulses of either nutrients or insecticides alone as predicted. Instead, the community response depended only on the presence or absence of insecticide pulses with no added or synergistic effect of nutrient pulses.

Communities responded similarly in both controls and N treatments. This result, while not predicted, was also not particularly surprising because while the N treatments received large pulses of nutrients, both treatments received the same environmentally realistic concentrations of nutrients over the course of the experiment. Experimental and theoretical work has shown that large pulses of nutrients can interfere with natural consumer-resource dynamics, for example by creating large fluctuations in phytoplankton abundance or shifts in phytoplankton edibility that could influence consumer effects on phytoplankton and allow plants to escape top-down control of herbivores (Holt, 2008; Scheffer et al., 2008; Cumming et al., 2013). In our experiment, the difference in delivery of nutrients between a more steady supply of small and regular inputs versus large pulses every two weeks appeared to have no significant effect on biological indicators over the course of the experiment. Therefore, the frequency and size of nutrient pulses themselves do not appear to have significant effects on freshwater communities, at least when average concentrations remain within "natural" expectable limits of an agricultural drain basin. This result may be a consequence of the fact that systems exposed to chronic disturbances may have reached a new stable state (Paine et al., 1998)

Communities that received insecticide pulses (I, NI, N_I), all showed similar responses that were significantly different from the control (C) and nutrient (N) treatments. The individual insecticide effects were independent of its application alone or in mixture with nutrients. This result agreed with studies that showed the effects of chlorpyrifos in nutrient enriched systems and it appeared to be independent of its mixture with nutrients (Van Donk et al., 1995). The same conclusion was reached by Cuppen et al. (1995) where chlorpyrifos caused an adverse direct effect on the zooplankton community under a combined exposure of insecticide with nutrients.

Insecticide treatments negatively affected zooplankton community structure from the first application as reflected by all biological indicators. Zooplankton abundance decreased under a chlorpyrifos concentration of 2 µg L^{-1} in our experiment, agreeing with previous observations that as little as 1 µg L^{-1} chlorpyrifos can have negative effects on zooplankton (Van Wijngaarden et al., 2005). In addition, there was a community shift from a cladoceran-dominated community in controls and N treatments to a copepod-dominated community in treatments with insecticide (I, NI, N_I). This general shift towards copepod-dominated communities has also been found in other studies (Hanazato, 1998, 2001; Relyea, 2005). Cladocera have been shown to be more sensitive to insecticides than copepods (Day et al., 1987; Hanazato, 2001). Additionally, this result was also expected since a model cladoceran species *Daphnia magna* has a documented LC50 of 1 µg L^{-1} for chlorpyrifos (Moore et al., 1998). The insecticide concentrations we used are within legal limits established to ensure no permanent environmental hazard and to allow for recovery, therefore recovery trends could have been expected in our experiment. We did not observe any trend of recovery of the zooplankton community between pulses of insecticide, suggesting that pulses every two weeks is frequent enough to prevent recovery. Our results agree with Brock et al. (2000) review on insecticides where a signal of recovery is only expected after two months of the last application if the exposure is lower than (0.1-1) x EC50 of the most sensitive standard test species.

The focus of our study was to observe how pulses of agrochemical mixtures affect freshwater food webs through both direct effects on species and indirect effects mediated through trophic interactions. Understanding the direct toxic effects based on toxicity from single-species is crucial for predicting both potential interactions (addition, antagonisms and synergisms) and indirect effects. For example, in our experiment the difference in sensitivity to insecticides between cladoceran and copepods allows copepods to survive and exploit the empty niche. The enhanced performance of copepods in the presence of insecticides is likely an indirect effect due to the release from food competition as cladocerans declined (Hanazato, 2001; Van Wijngaarden et al., 2005). The response of Chl *a* to treatments is also consistent with both direct and indirect effects. As expected, Chl *a* was highest in the treatments I , NI and N_I, suggesting that a release from grazing pressure from zooplankton affected by the insecticide was higher than the a priori positive effect of nutrients leading to an increase of phytoplankton. A similar pattern has been observed in other studies working with fungicides (Cuppen et al., 1995; Van den Brink et al., 2000) and biocides (Jak et al., 1998; Lin et al., 2012). These indirect effects of reduced grazing pressures, detected through significant increases of Chl *a* concentrations together with increases of dissolved oxygen and pH levels, are of extreme importance as warning signal of potential eutrophication impacts (Van Wijngaarden et al., 2005; Hanazato, 1998; Fleeger et al., 2003). In addition to phytoplankton abundance changing due to indirect effects of reduced grazing pressure, insecticides could also have caused phytoplankton shifts towards species that are inedible for specific taxa, such as cladocerans. It is known that the quality of food resources is important for reproductive cycles and development stages for zooplankton taxa (Caramujo and Boavida, 1999).

These results highlight that apart from direct negative toxic effects, indirect effects occurs via trophic interactions and these indirect effects can be as important as the direct effects in determining the food web response to disturbances. Therefore, more ecologically realistic ecotoxicological studies should be developed what could be understand as we attempt to move towards high-tier risk assessments or a strategy of multiscale experiments combinations. Complex experimental designs can make mechanisms more difficult to determine and interpretations more challenging due to the complexity of both population and community structure and trophic relationships (De

Meester et al., 2005). However, these types of studies allow a more comprehensive study of the response of an aquatic community that can incorporate both direct and indirect effects (e.g. Downing et al., 2008; Relyea and Hoverman, 2006), and will ultimately be necessary to increase the ecological realisms of risk assessments resulting in an improvement to prevent impacts in natural ecosystems.

CONCLUSIONS

This study was designed to gain a deeper understanding about how diverse agrochemical mixtures and input patterns affect non-target organisms in wetlands communities at realistic environmental concentrations. This approach will be crucial to increase the ecological realisms of risk assessment, and will be needed to set appropriate regulations that can meet population and ecosystem protection goals. Our results show that the effects of mixtures of chlorpyrifos and nutrients under these concentrations mixtures were not additive, antagonistic or synergistic, perhaps due to the high insecticide concentrations chosen. Nevertheless, indirect effects were detected and highlight the need of more ecologically relevant risk assessments to understand complex scenarios.

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CHAPTER 6

“Effects of intra- and interspecific competition on the sensitivity of aquatic macro-invertebrates to carbendazim”

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Effects of intra- and interspecific competition on the sensitivity of aquatic macro-invertebrates to carbendazim

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ABSTRACT

The Ecological Risk Assessment (ERA) of pesticides and other potentially toxic chemicals is generally based on toxicity data obtained from single-species laboratory experiments. In the field, however, contaminant effects are ubiquitously co-occurring with ecological interactions such as species competition and predation, which might influence the sensitivity of the exposed individuals to toxicants. The present study investigated how intra- and interspecific competition influence the response of sensitive aquatic organisms to a pesticide. For this, the effects of the fungicide carbendazim were assessed on the mortality and growth of the snail *Bithynia tentaculata* and the crustacean *Gammarus pulex* under different levels of intraspecific and interspecific competition for food, the latter being created by adding individuals of *Radix peregra* and *Asellus aquaticus*, respectively. The combination of competition and carbendazim exposure significantly influenced *B. tentaculata* growth, however, combined effects on survival and immobility were considered transient and were less easily demonstrated. Positive influence of competition on *G. pulex* survival was observed under low-medium carbendazim concentrations and under medium-high density pressures, being partly related to the enhancement of cannibalistic and predation compensation mechanisms under food limiting conditions. This study shows that intra- and interspecific competition pressure may influence the response of sensitive aquatic organisms in a more complex way (no, positive and negative effects were observed) than just increasing the sensitivity of the studied species, as has generally been hypothesised.

Keywords: competition, pesticides, population ecotoxicology, ecological risk assessment, carbendazim

INTRODUCTION

Pesticides used in agriculture production constitute one of the most important sources of anthropogenic pollution into aquatic ecosystems (Parra *et al.*, 2005; Stendera *et al.*, 2012). Currently, the Ecological Risk Assessment (ERA) of pesticides is mostly based on data obtained from single-species toxicity tests. Such an approach does not take into account ecological interactions between aquatic populations such as competition or predation (Van den Brink, 2013; Wootton, 2002; Brooks *et al.*, 2009) and may, therefore, underestimate or overestimate pesticide risks for sensitive species and for the structure of aquatic communities (Barata *et al.*, 2002; Beketov and Liess, 2005, 2006; Pestana *et al.*, 2009; Foit *et al.*, 2012; Knillmann*et al.*, 2012; De Laender and Janssen 2013). For instance, Beketov and Liess (2006) studied the influence of simulated predation on *Artemia* sp. populations exposed to the insecticide esfenvalerate. They concluded that the vulnerability of *Artemia* sp. populations affected by predation is considerably higher as compared to the populations that were not affected by predation since the population regulation capacity, measured as an increase in the production of offspring at low densities, was significantly reduced. Gui and Grant (2008) explored the responses of *Drosophila melanogaster* populations to toxicants and different food availability treatments. Results of their study indicated synergistic food-toxicant effects, but also indicated that compensatory mechanisms produced by toxicant exposure can occur at specific high competition levels due to density-dependent population processes. To date, only a few studies have investigated the combined effects of toxicants and ecological interactions on the sensitivity of aquatic organisms. Such studies are crucial to design more ecologically relevant ERAs (Linke-Gamenick *et al.* 1999; Gui and Grant, 2008;

Foit *et al.*, 2012; De Laender *et al.*, 2013) as well as to decide on whether or not, and under which conditions, the data generated by these studies should be incorporated in the intermediate tiers of ERA (e.g. De Laender *et al.*, 2008) and/or into ecological models used to underpin decisions during the risk assessment process (De Laender and Janssen, 2013; Van den Brink, 2013).

The main objective of the present study was to assess how intra- and interspecific competition affects the sensitivity of aquatic organisms to pesticide exposure under laboratory conditions. For this, two experiments were performed using the fungicide carbendazim and different levels of intra- and interspecific competition. These experiments represent an ecological scenario in which the studied species compete for a food resource while being exposed to a pesticide. The intraspecific experiments were performed by exposing different densities of the same species to a toxicant while the interspecific experiments were performed with two species, named as focal and competing species. The selected focal species were expected to show a higher sensitivity to carbendazim compared to the competing species, which allows to establish asymmetries on the food competition process and to better observe the combined effects of the pesticide and the competition stress on the focal species. The first experiment was performed using the snail *Bithynia tentaculata* as the focal species and the snail *Radix peregra* as the competing species. The second experiment was performed using the amphipod *Gammarus pulex* as the focal species, and the isopod *Asellus aquaticus* as the competing species. These aquatic taxa were selected because of their high abundance in aquatic ecosystems and their important ecological functions. For instance, snails account for up to 20%-60% of the biomass of macroinvertebrates in some freshwater ecosystems

(Habdija *et al.*, 1995), and amphipods crustaceans such as *Gammarus* sp. are considered crucial for ecosystem functioning due to their contribution to leaf decomposition (Petersen and Cummins, 1974; Zubrod *et al.* 2010).

MATERIAL AND METHODS

Test organisms

B. tentaculata and *R. peregra* were selected based on their difference in sensitivity to carbendazim. According to Cuppen *et al.* (2000) and the results of a preliminary test (results not shown), *B. tentaculata* was expected to be more sensitive than *R. peregra*. In addition, their co-occurrence in natural drainage ditches was also an important factor for the selection of this species combination. Snails were collected from Dutch drainage ditches and ponds and acclimatized to the same laboratory conditions as in the experimental set-up (see section 2.2). Only organisms in juvenile life stages and with similar length were selected (*B. tentaculata*: 5.4 ± 0.7 mm; *R. peregra*: 5.0 ± 0.7 mm).

G. pulex and *A. aquaticus* were also chosen due to their differences in sensitivity to carbendazim, with *G. pulex* being more sensitive than *A. aquaticus* (Van Wijngaarden *et al.*, 1998). Organisms were collected from a freshwater pond (Duno Pond, Renkum, The Netherlands, 51°58'9.31''N, 5°48'9.88''E) and acclimatized to laboratory conditions for 7 days prior to the start of the experiment. Only young adults with similar length were selected for the experiment (*G. pulex*: 10.6 ± 0.5 mm; *A. aquaticus*: 7.7 ± 0.3 mm).

Experimental set-up

Each experiment consisted of sixty glass jars of 1.5 L filled with 1 L of non-polluted pond water, previously filtered through a phytoplankton net (20 µm). The jars were placed in a water bath with a constant water temperature (20 ± 0.5 °C). High pressure metal halide lamps (Philips HPI-T, 400 W) were used to provide a daily photoperiod of 12 h, with a light intensity of approximately 500 $\mu E/m^2 \cdot s$ at the jar's water surface. In the experiment performed with *G. pulex* and *A. aquaticus*, a stainless mesh was added to each jar in order to increase the available surface and to serve as a refuge for the test organisms.

Both experiments consisted of a pre-treatment period, in which intra- and interspecific competition was allowed to take place, and an steady state exposure period, in which the combined effects of food competition and carbendazim exposure were evaluated. The experiments were performed in triplicate (n = 3), with five levels of species competition (i.e., control, medium and high intraspecific competition, medium and high interspecific competition) and four carbendazim treatments (i.e., control, low, medium and high exposure concentrations). In both experiments, competition levels and carbendazim treatments were randomly assigned to the test jars.

In the experiment performed with *B. tentaculata*, 0.59 ± 0.05 g of cucumber harvested from an organic farm was used as food resource. The amount of food provided to each jar was the amount consumed by 5 snails during one week in a preliminary feeding rate test. Cucumber was added weekly to the jars. Cucumber leftovers were removed from the jars before adding the next cucumber piece. In this experiment, the competition controls (C) consisted of jars stocked with 5 individuals of *B. tentaculata*. The jars corresponding to the medium (intra-M) and high (intra-H) levels of intraspecific competition were stocked with 10 and 20 individuals of *B. tentaculata*, respectively. The medium (inter-M) and high (inter-H) interspecific competition treatments were established by adding 5 and 10 individuals of *R. peregra* to jars containing 5 individuals of *B. tentaculata*, respectively. The competition was allowed to take place during 21 days (pre-treatment period) prior to the carbendazim exposure. Carbendazim was applied once a week for three weeks at a concentration of 400, 800 and 1200 µg/L to the low, medium and high exposure treatments, respectively. These exposure concentrations were selected based on the results of the microcosm study performed by Cuppen *et al*. (2000), who found a chronic NOEC (abundance) of 33 µg/L carbendazim for the focal species, *B. tentaculata*.

In the experiment performed with *G. pulex*, 0.7 ± 0.1 mg of pre-dried poplar leaves (*Populus* sp.) were used as competing food resource. This amount was based on the results of a preliminary feeding rate test and represents the amount consumed by 5 individuals of *G. pulex* per week. The competition controls (C) consisted of five individuals of *G. pulex*. The medium (intra-M) and high (intra-H) levels of intraspecific competition were established with a *G. pulex* density of 10 and 15 individuals per jar, respectively, and the medium (inter-M) and high (inter-H) levels of interspecific competition were set by adding 5 and 10 individuals of *A. aquaticus* to jars containing 5 *G. pulex* individuals, respectively. In this experiment, the pre-treatment period had duration of 4 days, and the exposure period lasted for 21 days. Carbendazim was applied once at a concentration of 20, 40 and 80 µg/L to the low, medium and high exposure treatments, respectively. These exposure concentrations were expected to approximate the LC10-LC50 range of the dose-response curve for *G. pulex*, based on previously published carbendazim toxicity data: acute (2d) LC_{10} and LC_{50} for juvenile *G. pulex* are 27 and 77 µg/L, respectively, while the chronic (21d) values for adults are 10 and 16 µg/L, respectively (Van Wijngaarden *et al.*, 1998). In both experiments, the effects of competition and carbendazim exposure were assessed on the mortality and growth of the focal (*B. tentaculata* and *G. pulex*) and the competing species (*R. peregra* and *A. aquaticus*). Mortality was monitored weekly in both experiments. Mortality of snails was assumed when they did not react after providing tactile stimuli on the soft part of their body with a laboratory needle. Mortality of *G. pulex* and *A. aquaticus* was assumed when they did not respond to any tactile stimuli. In both experiments, dead individuals were removed from the experiment. Snail and crustacean growth was quantified by measuring the relative changes in their shell or body length over time, respectively. Growth was assessed weekly in the experiment performed with snails. In the experiment performed with crustaceans growth was only measured at the end of the experiment to prevent overstress. In both experiments, growth was measured by taking high resolution pictures of the organisms in each jar (Microsoft LifeCam Studio) and analysing them with AxioVision SE64 Rel. 4.8. After the growth measurements were taken, the organisms were returned to their original jar. Additionally, the immobility of the snails *B. tentaculata* and *R. peregra* was evaluated at the end of the exposure period (day 21). For this, the snails were individually placed in a circular area of 5 mm radius drawn on the surface of a Petri dish containing unpolluted water. Immobility was assumed when they did not move out of the drawn circle after a time span of 30 minutes.

During the experiments, temperature, pH, dissolved oxygen concentration and conductivity were measured weekly at the start of the photoperiod. Temperature, pH and dissolved oxygen concentration were measured with a WTW 340i multi-meter, and conductivity was measured with a WTW 315i meter. These data were used to rule out any potential effect of water quality differences on the evaluated biological endpoints.

Carbendazim application and analysis

Carbendazim stock solutions (100 mg ai/L) were prepared using Derosal (50% carbendazim, w/v). Aliquots of the carbendazim stock solution were applied to the water surface of the jars of the carbendazim treatments and gently stirred with a laboratory spoon to ensure an homogeneous distribution of the pesticide over the water column. In the experiment performed with *B. tentaculata*, the test medium was renewed once a week to avoid excessive water quality deterioration. In the experiment performed with *G. pulex*, carbendazim was applied only once at the start of the exposure period. Because of the high reported persistence of carbendazim in water under laboratory conditions (Van Wijngaarden *et al.*, 1998), no additional carbendazim addition was deemed necessary to maintain the nominal concentration during the experimental period.

Water samples of 2 mL were taken from the jars after carbendazim addition to verify the nominal exposure concentrations and also at the end of the week to assess its dissipation (see Table I). A Perkin Elmer LC-90 UV detector was used to perform a direct analysis of the carbendazim concentrations in these samples. The mobile phase used was methanol:water (70:30), pumped at a flow rate of 0.7 mL/min with a Waters M590 pump through a Waters Novapak C-18 column. This column was set in a Waters Temperature Control Module at 40°C and with a wavelength of 285 nm. The retention time for carbendazim was 5 min. Calculation of the concentrations was based on external standard samples. According to this method, the limit of detection was 2 µg/L.

Table I. Measured carbendazim concentrations in the experimental medium and calculated Average Exposure Concentration (AEC) during the whole exposure period. The reported concentrations for the experiment with *B. tentaculata* correspond to the measured concentrations after the first carbendazim addition (0d), before the second carbendazim pulse (7d), and seven days after the second pulse (14d). The reported concentrations for the experiment with *G. pulex* correspond to the measured concentrations after the carbendazim application (0d), and 7 (7d) and 14 (14d) days after the application. Concentrations are expressed as mean ± standard deviation (µg/L). n.m.: not measured.

Experiment	Nominal concentration	Measured concentrations				AEC (Day 0 – 14)
		0d	7d	14d	21d	
B.tentaculata	400	374 ± 2	379 ± 2	366 ± 5	n.m.	373 ± 2
	800	753 ± 2	734 ± 4	756 ± 2	n.m.	749 ± 2
	1200	1127 ± 6	1105 ± 10	1122 ± 3	n.m.	1119 ± 4
G. pulex	20	22 ± 1	15 ± 1	n.a.	n.m.	21 ± 1
	40	41 ± 1	37 ± 2	42 ± 1	n.m.	40 ± 1
	80	79 ± 1	72 ± 1	69 ± 1	n.m.	78 ± 1

Statistical analyses

The effects of the species competition treatments on the sensitivity of the focal species to carbendazim was assessed by (1) comparing the calculated LC50 or EC50's between competition treatments for each sampling day, and (2) by using Generalized Linear Models (GLMs). The calculation of the EC50 and LC50 values was carried out by means of log-logistic regression using the software GenStat 11th (VSN International Ltd., Oxford, UK), as described by Rubach *et al.* (2011). EC50s were calculated for the growth of *B. tentaculata* at the end of the experiment (day 21) and LC50s were calculated for *B.*

tentaculata and *G. pulex* at each sampling day. The GLM analysis was performed for each measured endpoint at each sampling day using the same software (GenStat 11th). The model used for the GLM analysis was adapted to the data distribution of the different measured endpoints. Immobility and survival were assessed using a binomial distribution and logit as the link function, while growth was evaluated by using a Poisson distribution and logarithm as the link function. The statistical model was defined by a constant, the exposure concentration, the competition level and their interaction, introducing both, the nominal pesticide concentrations and the competition treatments, as groups. The effects of the pesticide concentration, the competition level or the combination of both on the evaluated biological endpoint were considered to be significant when the calculated p-values were < 0.05, and were defined as moderately significant when they were between 0.05 and 0.10.

RESULTS AND DISCUSSION

Carbendazim concentrations and water quality

During the whole experiment the average measured carbendazim concentrations were 91.3 ± 13.9 % and 100 ± 16.3 % of the nominal concentrations for the *B. tentaculata* and the *G. pulex* experiments, respectively. In line with previous studies (Van Wijngaarden *et al.*, 1998; Slijkerman *et al.*, 2004), carbendazim was found to be very stable during the experimental period, with an average 7-day dissipation rate of 1.8 ± 0.3 % in the *B. tentaculata* experiment, and 13.6 ± 1.6 % in the *G. pulex* experiment (average ± SD, Table I). There were no observable effects of the carbendazim exposure concentration or the organism density on the water quality parameters measured during the course of the experiments. The average values of the measured water quality parameters were: temperature 20.9 ± 0.5 ºC, pH 8.0 ± 0.5, dissolved oxygen 7.9 ± 1.9 mg/L, and conductivity 781 ± 54 µS/cm (average ± SD).

Toxic effects of carbendazim

In the *B. tentaculata* experiment, significant differences in mortality between controls and the carbendazim treatments were only found on day 21 (Table II). However, the measured mortality rate on that sampling day was not high enough to fit a dose-response model and an LC50 could not be calculated. Carbendazim exposure resulted in significant effects on *B. tentaculata* mobility on day 21 (Table II). The calculated EC50-21d (immobility) value was < 373 µg/L (Table III). In contrast, Van Wijngaarden *et al.* (1998) calculated a higher EC50-28d (immobility) of 1641 µg/L (1169 – 2303) µg/L. Discrepancies between our results and those reported by Wijngaarden *et al.* (1998) could be partially related to the differences in immobility definition and feeding regime; i.e., in the experiment of Wijngaarden *et al.* (1998) immobility was assess as the absent of response of any kind after 30 seconds as a result of tactile stimulation whereas in our experiment immobility was consider the absent of movement out of a drawn circle after a time span of 30 minutes; and, animals were fed *ad libitum* by Van Wijngaarden *et al.* (1998) whereas in our experiment they had severe food restrictions. Significant effects on growth could, however, not be demonstrated in none of both competition experiments (Table II, Fig 1b, 1d). Low energy input under toxic stress could affect important vital traits of snails such as mobility or feeding behaviour in an attempt to optimize the new energetic balance. For example, Tripathi and Singh (2002), found a decrease in the glycogen concentration in snail tissues exposed to pesticides, which was attributed to the mobilization of this substance to meet the high energy demands required to mitigate toxic stress (Tripathi and Singh, 2002). No LC50 or EC50 which was within the tested concentration range could be calculated for the non-focal (*R. peregra*) species (Table A, supplementary material).

Table A. Calculated LC50 and EC50 values for the competing species. The sampling days that are missing in the table did not show concentration-response or the LC50 could not be calculated. Inter-M: medium interspecific competition; inter-H: high interspecific competition. n.m.: not measured

Species		LC50	EC50 (immobility)
		48h	21d
Radix peregra	Inter-M	>373	> 373
	Inter-H	> 373	1482 (300 – 7313)
Asellus aquaticus	Inter-M	80 (-)	n.m.
	Inter-H	>80	n.m.

Significant effects of carbendazim on *G. pulex* mortality were observed on day 7, 14 and 21 after the start of the exposure period (Table II, Figure 2a). LC50 values were calculated for all sampling days, except for day 2, for which mortality was not high enough to fit a dose-response model (Table III). The LC50-96h for *G. pulex* in the competition control, 71 (36-139) µg/L, was found to be similar to the LC50-96h value reported by Van Wijngaarden *et al.* (1998) for *G. pulex* juveniles: 55 (41 – 75) µg/L. The calculated LC50 values for the competition control on day 7, 14 and 21 (Table III) also fall within the LC50 95% confidence intervals reported by Van Wijngaarden *et al.* (1998) for the same exposure periods, confirming the previously reported sensitivity of this species to carbendazim. Analyses of carbendazim impacts on *G. pulex* growth on day 21 could not be carried out due to the elevated mortality and the consequent insufficient amount of available data points. No LC50 or EC50 which was within the tested concentration range could be calculated for the non-focal (*A. aquaticus*) species (Table A, supplementary material).

Table II. Results of the GLM analysis (p-values) showing the effects of the carbendazim treatment, the competition level, and their combination on mortality, immobility and growth for *B. tentaculata* and *G. pulex* along the experiment. Bold values indicate significant (p<0.05) or marginally significant (0.05 ≤ p ≤ 0.1) effects. n.m.: not measured. n.c.: not calculated. Not enough mortality to fit the dose-response model. n.e.: not evaluated. Mortality was too high and effects on growth could not be evaluated.

<table>
<tr><th colspan="2" rowspan="3">Experiment</th><th rowspan="3">Independent variable</th><th colspan="9">Days/Endpoint (p – values)</th></tr>
<tr><th colspan="5">Mortality</th><th>Immobility</th><th colspan="3">Growth</th></tr>
<tr><th>2</th><th>4</th><th>7</th><th>14</th><th>21</th><th>21</th><th>7</th><th>14</th><th>21</th></tr>
<tr><td rowspan="6">B. tentaculata</td><td rowspan="3">Intraspecific competition</td><td>Carbendazim</td><td>0.99</td><td>0.47</td><td>0.44</td><td>0.30</td><td>0.001</td><td>< 0.001</td><td>0.39</td><td>0.56</td><td>0.65</td></tr>
<tr><td>Competition</td><td>0.27</td><td>0.40</td><td>0.74</td><td>0.87</td><td>0.27</td><td>0.81</td><td>< 0.001</td><td>0.01</td><td>0.01</td></tr>
<tr><td>Combined</td><td>0.02</td><td>0.001</td><td>0.13</td><td>0.60</td><td>0.95</td><td>0.70</td><td>0.16</td><td>0.34</td><td>0.87</td></tr>
<tr><td rowspan="3">Interspecific competition</td><td>Carbendazim</td><td>0.01</td><td>0.23</td><td>0.44</td><td>0.20</td><td>0.03</td><td>< 0.001</td><td>0.64</td><td>0.72</td><td>0.28</td></tr>
<tr><td>Competition</td><td>0.05</td><td>1.00</td><td>0.01</td><td>0.12</td><td>0.31</td><td>0.29</td><td>0.08</td><td>0.02</td><td>0.02</td></tr>
<tr><td>Combined</td><td>0.10</td><td>0.06</td><td>0.97</td><td>0.30</td><td>0.82</td><td>0.29</td><td>0.93</td><td>0.06</td><td>0.44</td></tr>
<tr><td rowspan="6">G. pulex</td><td rowspan="3">Intraspecific competition</td><td>Carbendazim</td><td>n.c.</td><td>n.c.</td><td>< 0.001</td><td>< 0.001</td><td>< 0.001</td><td>n.m.</td><td>n.m.</td><td>n.m.</td><td>n.e.</td></tr>
<tr><td>Competition</td><td>n.c.</td><td>n.c.</td><td>0.78</td><td>0.78</td><td>0.29</td><td>n.m.</td><td>n.m.</td><td>n.m.</td><td>n.e.</td></tr>
<tr><td>Combined</td><td>n.c.</td><td>n.c.</td><td>0.254</td><td>0.255</td><td>0.06</td><td>n.m.</td><td>n.m.</td><td>n.m.</td><td>n.e.</td></tr>
<tr><td rowspan="3">Interspecific competition</td><td>Carbendazim</td><td>0.06</td><td>0.001</td><td>< 0.001</td><td>< 0.001</td><td>< 0.001</td><td>n.m.</td><td>n.m.</td><td>n.m.</td><td>n.e.</td></tr>
<tr><td>Competition</td><td>0.97</td><td>0.83</td><td>0.09</td><td>0.01</td><td>0.01</td><td>n.m.</td><td>n.m.</td><td>n.m.</td><td>n.e.</td></tr>
<tr><td>Combined</td><td>0.29</td><td>0.55</td><td>0.89</td><td>0.47</td><td>0.47</td><td>n.m.</td><td>n.m.</td><td>n.m.</td><td>n.e.</td></tr>
</table>

Table III. LC50 and EC50 values and their 95% confidence intervals for *B. tentaculata* and *G. pulex* calculated for each competition treatment level. Concentrations are expressed in µg/L. C: control; intra-M: medium intraspecific competition; intra-H: high intraspecific competition; inter-M: medium interspecific competition; inter-H: high interspecific competition. [a] Due to the absence of a concentration having a partial effect, no EC50 could be calculated for the control treatment. n.c.: not calculated. Dose-response model could not be fitted.

Experiment	Endpoint	Day	Control (C)	Intraspecific competition		Interspecific competition	
				intra-M	intra-H	inter-M	inter-H
B. tentaculata	EC50 (immobility)	21	< 373[a]	342 (233 – 502)	137 (34 – 551)	391 (192 – 769)	124 (3 – 5146)
G. pulex	LC50	4	71 (36 – 139)	74 (49 – 110)	n.c.	n.c.	115 (27 – 497)
		7	30 (22 – 41)	n.c.	n.c.	38 (37 – 39)	25 (19 – 33)
		14	30 (22 – 40)	38 (29 – 48)	34 (27 – 42)	38 (37 – 38)	17 (14 – 20)
		21	22 (17 - 30)	31 (22 - 44)	22 (17 - 29)	37 (36 - 37)	18 (17 - 19)

Single and combined effects of competition and carbendazim

B. tentaculata experiment

Significant effects of intraspecific competition on mortality and immobility were generally not detected (Table II, Figure 1a). However, intraspecific competition significantly affected growth rates of *B. tentaculata*, indicating that competition over resources was present (Table II, Figure 1c). Due to the low carbendazim effects on mortality observed in this experiment, LC50s could not be calculated for the different competition treatments (Figure 1a, Table III). However, the dose-response patterns on immobility showed that the EC50 values for the competition controls and for the intermediate intraspecific competition treatment were approximately two times higher than the ones calculated for the highest competition treatment, indicating that high competition under pesticide exposure could result in an increased snail immobility (Table III). Combined effects of intraspecific competition and carbendazim stress were only detected on the mortality endpoint at the start of the exposure period (Table II). Such effects were mainly appreciated at the low carbendazim treatment (400 µg/L) and did not show a consistent dose-response pattern (Figure 1a) as this interaction was not observed at the medium density treatment (Figure 1a). This observation is consistent with the proposed theory of toxicant-induced reduction of intraspecific adverse effects (Liess 2002). Liess (2002) studied the influence of intraspecific competition on a trichopteran (*Limnephilus lunatus* Curtis) population exposed to fenvalerate, and reported compensation of direct pesticide effects due to a reduction of indirect intraspecific pressure as compared to the competition controls. Such mechanism could explain the absence of increased mortality in the medium intraspecific competition treatment. Our results also suggest that after certain organism density threshold those effects are not compensated by low impact pesticide exposure and that the combined effects of pesticide and competition stress are better appreciated at low-medium individual density and exposure stress. In addition, an EC50 could not be calculated in the control treatment (C), although the observed response was very similar to the high intracompetition treatment (intra-H), and to a lesser, extent to the medium intracompetition (intra-M) (Table III).

Interspecific competition slightly influenced *B. tentaculata* growth both positively and negatively (Table II, Figure 1d) and significantly increased mortality on day 2 and 7 after the start of the exposure period. Combined effects of competition and carbendazim exposure on mortality were only significant on day 4 (Table II), however a clear trend was observed towards higher mortality rates in the highest competition treatment during the whole experimental period (Figure 1b). Even though, mobility was not significantly affected by interspecific competition, nor by the combination of interspecific competition and carbendazim (Table II).

***G. pulex* experiment**

Although significant intraspecific competition effects on mortality were not detected at any sampling day for *G. pulex* (Table II), a clear trend towards increased mortalities at higher densities was observed in the carbendazim controls (Figure 2a). This trend is most likely related to higher rates of cannibalisms in the controls at higher densities which was not observed in the carbendazim treatments due to immobilisation (immobility endpoints were not measured). Combined effects of intraspecific competition and carbendazim stress on *G. pulex* mortality were found to be moderately significant at the end of the exposure period (Table II). Although calculated LC50s for sampling days 14 and 21 were similar between competition treatments (Table III), a reduced lethal effect of carbendazim at the medium and high intraspecific competition treatments was observed, particularly at the high carbendazim exposure treatment (Figure 2a). This reduced lethal effect at medium-high densities could be related to the *Gammarus* sp. cannibalism behaviour regulated by food scarcity pressure (Dick, 1995). Therefore, pre-treatment competition could have led to a decrease of the population abundance at both medium and high species density levels. Cannibalism at the intra-M competition levels seemed to compensate the effects of the toxicant on the surviving individuals, and could explain why mortality is lower than in the control and in the high density at the 20 and 80 µg/L treatment levels. On the other hand, at the intra-H competition level, the cannibalism did not seem to outweigh the combined effect of carbendazim exposure and competition based on the observation of equal rates of mortality between controls and intra-H competition levels.

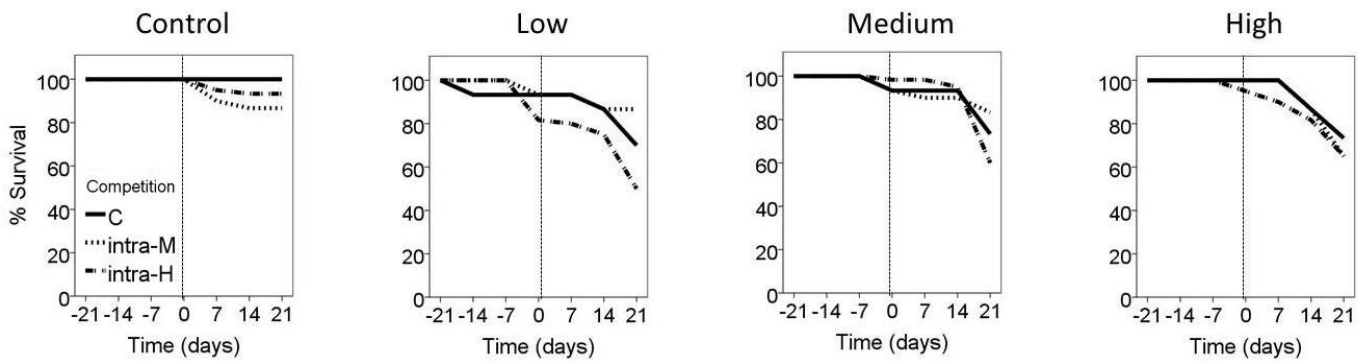
Interspecific competition between *G. pulex* and *A. aquaticus* significantly affected *G. pulex* survival, but no significant interaction between of interspecific competition and carbendazim exposure was observed on *G. pulex* mortality (Table II). The high interspecific competition treatment had a negative effect on the individuals survival at the 20 and 40 µg/L exposure levels (Figure 2b), as shown by the slightly lower LC50 for the high competition level (17 µg/L) as compared to the controls (30 µg/L) and the intermediate competition level (38 µg/L) for day 14 (Table III). The toxic effects of carbendazim on the *G. pulex* mortality in the intermediate competition level was assumed to be alleviated by the presence of *A. aquaticus,* since *G. pulex* is known to strongly predate on *A. aquaticus* (Blockwell *et al.*, 1998). This could be confirmed by the increased survival of *A. aquaticus* with increasing concentrations, probably because *G. pulex* predation rates were affected by the pesticide concentrations (Figure 4). This result demonstrated that competition stress combined with chemical exposures is able to influence species interactions. Our findings are in line with the study by Gui and Grant

(2008), who demonstrated that food availability could outweigh the toxic impact of chemicals on the dipteran *Drosophila melanogaster*.

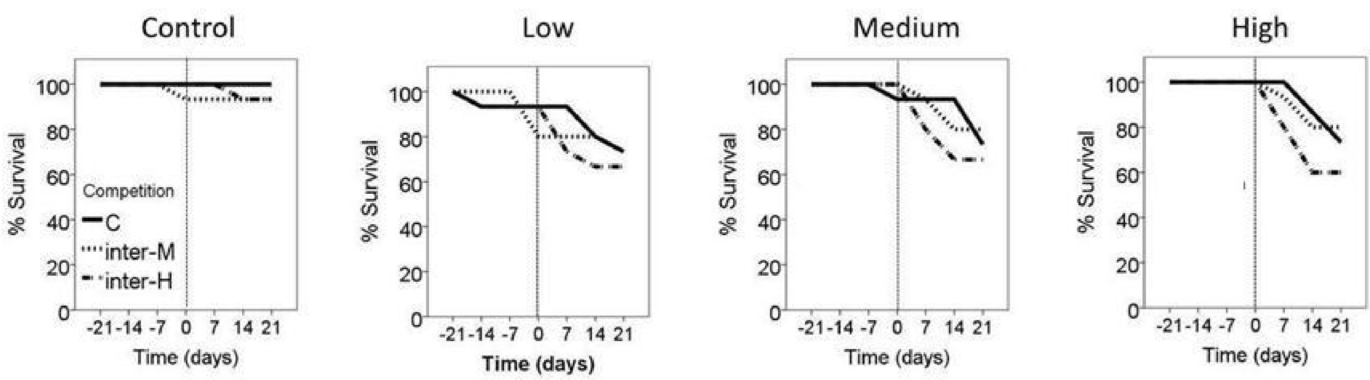
To sum up, the results of our experiments show the potential complexity of populations' responses under combined effects of competition and chemical exposure since the interaction of both pressures can vary depending on competition and toxicant concentration pressure levels. It was observed that at high interspecies competition pressure, the depletion of food availability combined with toxic effect cannot be compensated by predation benefits for *G. pulex* due to the presence of *A. aquaticus*. This could be a result of the higher density at initial conditions, which probably overstressed the population for food availability (inter-H, 5 *G. pulex vs.* 10 *A. aquaticus*; inter-M, 5 *G. pulex vs.* 5 *A. aquaticus*). The interactions between different levels of ecological interactions and the levels of toxicant exposure have previously been reported. For example, Linke-Gamenick *et al.* (1999) studied density-dependent effects of polycyclic aromatic hydrocarbons (PAHs) and of a fluoranthene (FLU) on survival, growth rate and reproduction of a polycahete (*Capitella* sp.) and found that at low food limitations and low toxicant concentrations the toxic effects were marginal, whereas at high toxicant concentration, food limitation intensified the toxic impacts (synergistic effects). This also corresponds with the study by Barata *et al.* (2002), who found that at medium limiting food resources the negative toxic effects on population abundance drastically increase with increasing animal density, suggesting that compensation of toxicant impacts is related to mortality driven by competition stress. On the contrary, several studies report negative effects of food limitation on species sensitivity. For instance, Stamplfli *et al.* (2011) found that the abundance of zooplankton species was more affected by a pesticide under food limited conditions. Foit *et al.* (2012) performed an experiment to test the competition effect on sensitivity and recovery capacity of two interacting populations (*Daphnia magna* and *Culex pipiens molestus*) and concluded that toxicant sensitivity was positively correlated to competition and delayed recovery. Therefore, there are evidences of both positive and negative combined effects of competition and toxicant effects. Effects of combined stressors (competition and pesticide) on mortality were expected since competition for food resources is one relevant ecological interaction acting at intra- and interspecific levels influencing mortality and development at individual and populations levels (Van Buskirk, 1987; Gordon, 2000). There are models and experimental evidence of the importance of competition on fitness responses under toxicant exposure, nevertheless such interactions have not been taken into account in ERA. For instance, Kooijman and Metz (1984) modelled toxicant effects on individual fitness (metabolism, feeding, survival) under different food competition pressures and showed larger impacts on species development under higher competition pressure levels. This agrees with the influence of competition (density-dependence factor base on food availability) detected on the survival response of both *B. tentaculata* and *G. pulex* under toxicant exposure.

Figure 1. Effects of carbendazim exposure and species competition on *B. tentaculata* survival and growth. The carbendazim exposure is represented as: Control, Low: 400, Medium: 800, and High: 1200 μg/L); C: control; intra-M: medium intraspecific competition; intra-H: high intraspecific competition; inter-M: medium interspecific competition; inter-H: high interspecific competition. The dashed vertical line indicates the start of the carbendazim exposure period.

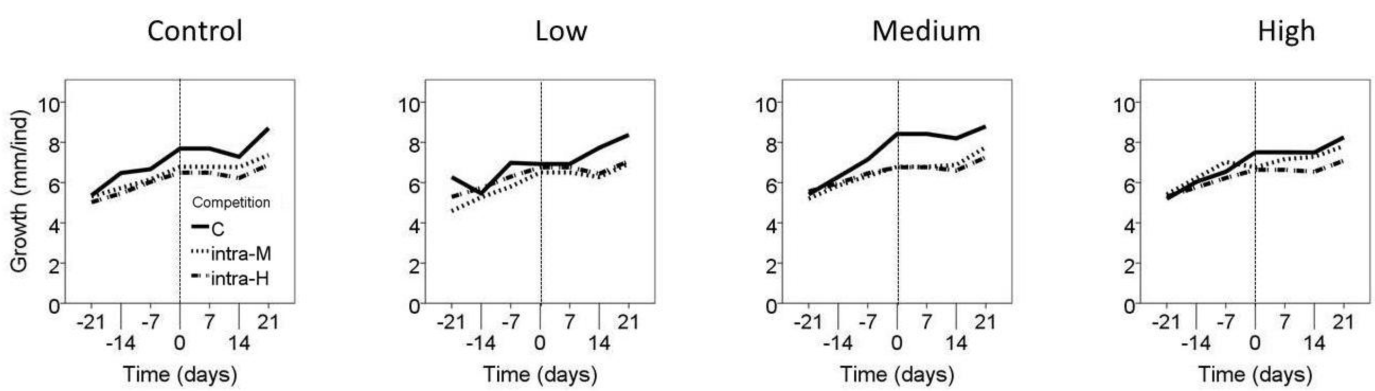
1a) Survival: intraspecific competition



1b) Survival: interspecific competition



1c) Growth: intraspecific competition



1d) Growth: interspecific competition

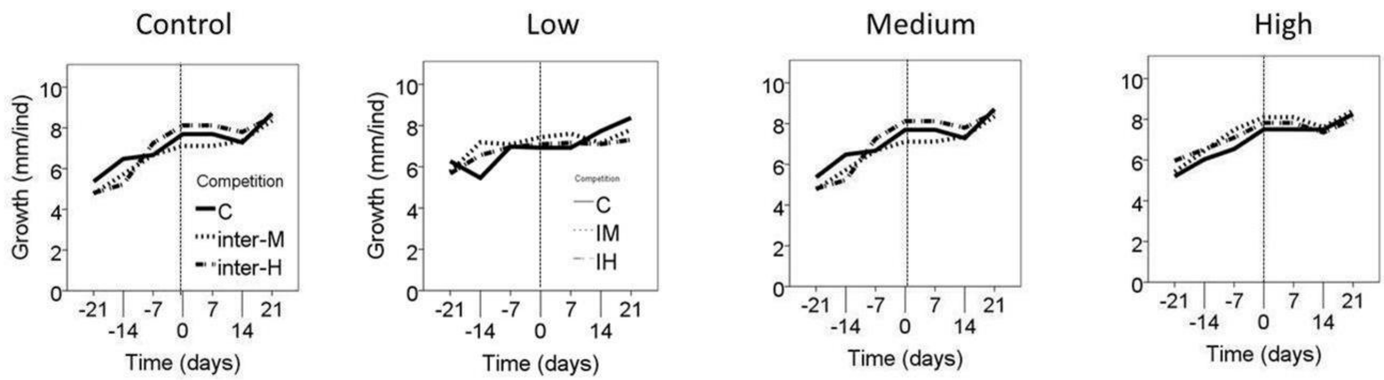
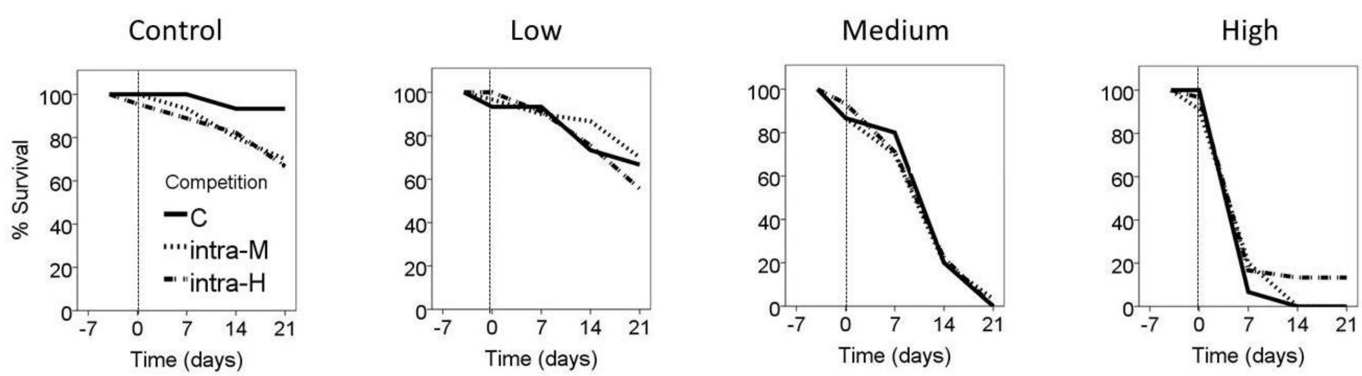


Figure 2. Effects of carbendazim exposure and species competition on *G. pulex* survival. The carbendazim exposure concentrations are represented as: Control, Low: 20, Medium: 40, and High: 80µg/L. C: control; intra-M: medium intraspecific competition; intra-H: high intraspecific competition; inter-M: medium interspecific competition; inter-H: high interspecific competition. The dashed vertical line indicates the start of the carbendazim exposure period.

2a) Survival: Intraspecific competition



2b) Survival: Interspecific competition

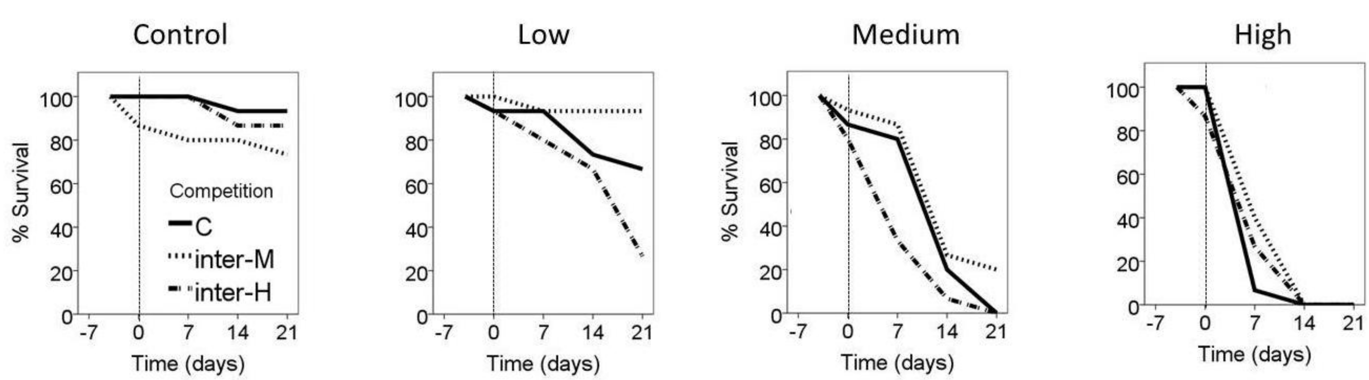
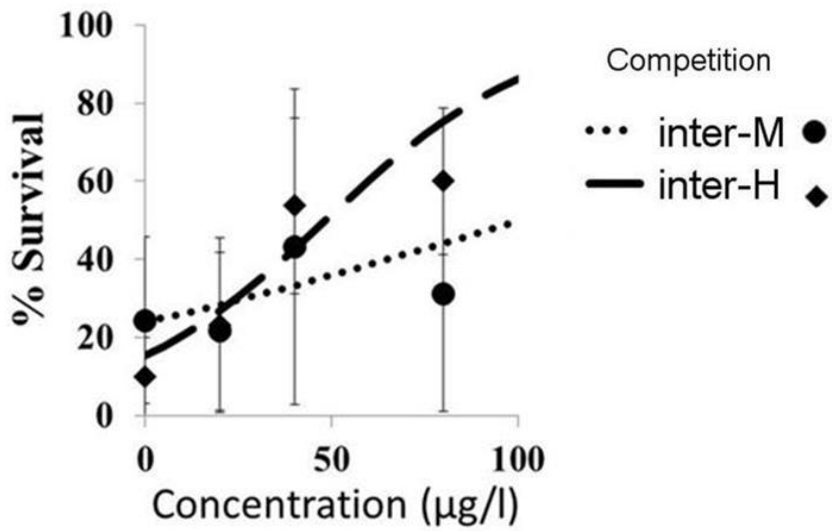


Figure 4. Probability of survival of *A. aquaticus* at the end (day 21) of the interspecific competition experiment with *G. pulex*.



The importance of ecological interactions for risk assessment

Ecological interactions such as competition and predation are highly relevant for population responses in the field, so excluding them from ERA may lead to inefficient (over or under protective) regulations at both economic and ecological levels. Although it has been generally assumed that competition enhances the negative effects of a toxicant (Foit *et al.*, 2012; Stamplfli *et al.*, 2011), some studies have shown that population-level effects of food limiting conditions under chemical stress could be outweighed under medium-high population densities and low toxicant concentrations (Gui and Grant 2008; Linke-Gamenick *et al.* 1999; Barata *et al.*, 2002). The results of the *G. pulex* experiment are consistent with the latter hypothesis and indicate behavioural changes as the main drivers determining the effects of competition. Here we observed that *G. pulex* increased its cannibalistic habits and increased predation on its competitor (*A. aquaticus*) under low toxicant exposure, thus outweighing the stress imposed by the food limiting conditions. Based on our results, we can conclude that the trend of the interaction effect (- or +) seems to depend on the ecology of the particular focal and competing species, the density of the focal and the competing species, and the toxicant pressure. The results of the experiments here presented support the need to include both ecological intra- and interspecific interactions in ERA to better understand the combined effect of ecological aspects and toxic disruption on aquatic communities by, for example, including them into food-web and meta-population models. They also show that, considering the high complexity in the observed responses, model developers should accumulate a greater amount of information to realistically introduce more ecology into ERA.

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DISCUSSION

Human population is predicted to continue growing what implies a higher demand of agricultural needs worldwide increasingly dependent on agrochemicals. Consequently, an intensification of fertilizers and pesticides use is expected under current agricultural practices. Therefore, it will raise the stressed linked to agricultural activities on already threaten natural systems as wetlands. If this scenario is not tackled, there will be objectives asymmetry between European Union (EU) sustainable development policies and real practices implemented in the field. The EU seeks to achieve a "sustainable development that meets the needs of thepresent without compromising the ability of future generations to meet their own needs" since the Brundtland Commission in 1987 (COM, 2001). It is the background for many directives with environmental protection goals and projects aiming to assess ecological risks, for instance, of agrochemicals as the topic we have targeted in this thesis. In this context of increasing pressures and wiliness to protect the environment, ecotoxicology has to face the challenge of increase its complexity in order to predict and reduce ecological risks. In fact, many voices claim the need of more complex and ecological relevant assessment of agrochemicals in order to achieve EU directive protection goals at ecosystem levels (De Laender *et al.*, 2013). Under such complex framework, this thesis has focused on two main scenarios: **agrochemical scenarios** (mixture, pulses frequency and limits) and **ecological scenarios** (ecological interactions: competition and hierarchical levels).

1. Legal limits and its protection capacity

Legal limits are established based on results from single species test. Despite the important information provided by single species tests, they lack environmental and ecological realisms to extrapolate their results to community or ecosystem levels.

Consequently, it raises doubts about the effectiveness of current legal limits to prevent ecological risk.

As expected, **Chapter 1, 2, 3 and 4** showed negative effects of agrochemicals (copper sulfate and ammonium nitrate) on plankton community even if exposure concentrations were within legal limits. In **Chapter 1** the community was exposed to copper concentration **below and above** legal limits. The community was adversely affected by both concentrations: phytoplankton community structure came out as an early signal endpoint owing to its fast response. Phytoplankton showed an abundance decrease and size structural changes towards small cell size classes what is identify as an impairment indicator; and, zooplankton community suffered an abundance decrease and taxa disappearance (copepods and rotifers). **In chapter 2** the community was exposed to two copper concentrations **within legal** limits: phytoplankton presented two diverse responses based on copper concentrations, surprisingly the low treatment (2 µg Cu l^{-1}) had a negative effect and the high treatment (20 µg Cu l^{-1}) did not, what highlights the complexity of community responses and the occurrence of indirect effects; and, zooplankton response was not significant even though a tendency of community change could be observed (higher presence of cladocera, copepoda and ostracoda *versus* rotifera and nauplii). In **chapter 3,** plankton community was treated with nitrate within legal limits: phytoplankton abundance decreased owing to negative effects of nitrate addition and later, increased as a result of indirect effects related to zooplankton community changes; and, zooplankton community was negatively affected suffering a decrease of abundance and a community change from cladocera-dominated to copepoda-dominated community. In **chapter 4**, plankton community changes as result of mixture of copper and nitrate within legal limits were studied: phytoplankton abundance increased in the treatments; and, zooplankton abundance fluctuated in treatments with copper while continuously increased in treatments with nitrate on the last sampling days. Community shifts are just observed in treatments with only copper resulting in an increase of *Ceriodaphnia ssp.* and rotifera. In summary, either permanent or transient adverse effects of agrochemical concentrations within legal limits have been observed in all experiments. These results support the need of moving from single species tests to more ecological realistic tests to establish legal limits of agrochemicals in field conditions. A complementary action could be a more conservative application of the precautionary principle. Inefficient legal limits consequences go far beyond direct ecological impacts. Ecosystems resilience is impaired by ecological impacts driven by routinely chemical exposures. Therefore, impacted ecosystems response capacity to local and global changes would decrease ecosystem services resulting on biodiversity losses which have social, economic and environmental negative consequences.

Community complexity in terms of diversity at community structural levels was higher in chapter 3 and 4 than in 1 and 2. Based on the functional redundancy hypothesis and the resilience hypothesis (Walker *et al.,* 1999), it means that if the dominant species are impaired by the chemical stress, the minor species would have been expected to substitute them and its functional role in the community. Therefore, the ecosystem resilience would be maintained being able to partially counterbalance the potential adverse chemical effects on the community. Communities' shifts after disturbances would occur based on this hypothesis. In all four experiments (Chapter 1, 2, 3 and 4) zooplankton community structural changes have been observed as a result of different group sensitivities and indirect effects due to phytoplankton changes. In the long term, it could mean either a disrupted community or a community able to recover. From a conservative point of view, even if the community is resilient and able to function similarly to an undisturbed community, it can be considered impacted since it differs from reference conditions. In addition, natural communities are exposed to routinely detected agrochemicals concentrations what could result on tolerance development to the stress though a) adaptation or acclimation responses at population-level, and b) shifts in species composition (Schmitt-Jansen *et al.,* 2008). Neither single species tests nor overall abundance indicators are able to detect these changes. Ecosystem responses are intricate and Science seeks to understand them, therefore, the mention redundancy hypothesis, the resilience hypothesis or others as the biodiversity-stability debate are the engine of progress. The translation of Science results into management practices may have uncertainties cluttering policy makers and management decisions takers. However, each new scientific experiment contributes to lower those uncertainties and to make more science-based decisions. Regarding the mentioned chapters, it can be stated that it is extremely important to assess community structural changes owing to its role in ecosystem resilience and stability influencing recovery capacity which is useful information for the decision process when establishing legal limits.

2. Mixtures and frequency of agrochemicals exposures: a step further into complexity. Dealing with uncertainty but gaining information.

Realistic field chemical exposures should include mixtures of chemicals and repeated applications. Most of the studies on mixtures of chemicals or disturbances focus on single species rather than communities in most of the cases under an only chemical pulse (Hurd *et al.,* 1996; Jonker *et al.,* 2005; LeBlanc *et al.,* 2012). Therefore, there is a lack of experiments assessing complex scenarios including mixtures and application timing at community levels. Such kind of experiments would allow detecting indirect effects that are missed in simple single species and chemical tests. In fact, it is known that mixtures effects differ from single effects owing to chemicals interactions leading to independent, antagonistic or synergistic responses (Lydy et al., 2004; Deener, 2000; LeBlanc *et al.*, 2012). In addition, community responses would vary depending on its dynamics; therefore, the timing of chemical events may have different effects at different periods (Hughes and Connell, 1999).

The aim of **chapter 4 and 5** was to contribute to the awareness of the need of new experimental design of scenarios providing data from varying mixtures and frequency exposures experiments. There are a nearly infinite set of possible mixtures and frequency scenarios, therefore, the selected ones were based on the most realistic exposures conditions in the areas where the experiments took place. In addition, these two chapters complement the claim of **Chapter 1, 2 and 3** of the need of more complex experiment to establish agrochemical legal limits. In **chapter 4,** the mixture of fertilizers (ammonium nitrate) and fungicide (copper sulfate) shows interaction between effects. It seems than in the mixture treatments the nutrients can counterbalance the direct toxic effects of copper on zooplankton community. This compensation may be mediated by a transient increase of phytoplankton so then more food availability favoring zooplankton fitness to face toxic adverse effects. Therefore, the comparison of single versus mixtures exposures in this experiment denotes a higher effect of single agrochemical exposures than mixture exposures which effects are mediated and counterbalance for indirect effect across the trophic web. In **chapter 5**, a different response is observed in mixtures and frequency exposures of nutrients (nitrate and phosphorus) and insecticide (chlorpyrifos). In this experiment, no compensation effects were observed in mixture versus single exposures. The increase of phytoplankton was not enhanced by nutrients availability but by a decrease of grazing pressure owing to direct and drastic effect of the insecticide on zooplankton. The conclusions about the comparison of single versus mixtures exposures from this experiment is that nutrients and insecticide toxic effects do not interact what is most likely owing to the drastic toxic effect of the insecticide. Nevertheless, the experiment allows identifying which of the toxic will be the harmful one when both co-occur. Frequency of treatments application was not relevant in this case because of the drastic effect of insecticide since the first pulse. However, frequency and timing of chemical application is a relevant factor because it would impact the community in different moments. Therefore, chemical exposures might disrupt different development and dynamic stages what would result in diverse community responses. This kind of information is crucial for managers to make science-based decision of agrochemical application periods.

The microcosms studies here presented have allowed to detected compensation responses and indirect effects on plankton communities exposes to different mixtures of agrochemicals. In terms of frequency, no influence on the community response was found because of the drastic effect of the agrochemical used since the first application. In order to record pulse frequency effects, an agrochemical with lower toxicity should have been used. However, there are previous studies pointing out the importance of pulses and its frequency. Communities exposed to constant pulses may develop some degree of tolerance as a response to the subletal continuous previous exposures (Johansson *et al.,* 2001; García-Muñoz *et al.*, 2011); while, communities under less frequent but more intense pulses suffer from more stress owing to those drastic changes (Earl and Witheman, 2009; García-Muñoz *et al.*, 2011). It highlights the complexity of communities' responses and its dependence on mixtures composition and application frequency. Therefore, the development of agrochemical application policies and the establishment of legal limits will be more appropriate considering mixture and frequency factors what increase the environmental realisms of ecological risk assessments. Even though, it will also increase the uncertainties and make effects less detectable compare to single agrochemical exposures when the possibility of interaction is given weight (European Commission 2006, project NoMiracle). It was the case in **chapter 4 where** the mixture of fertilizers (ammonium nitrate) and fungicide (copper sulfate) resulted in lower changes on zooplankton abundance owing to indirect effect of nutrients able to slightly counterbalance the direct copper toxic effect. In any case, the community was affected because it differed from the reference conditions. Therefore, the relevance of this result is the need to establish larger safety factors to face the higher complexity of more environmental realistic scenarios.

3. Combined effect of ecological and chemical aspects

Efforts pursuing to increase environmental realisms of Ecological Risk Assessment include the consideration of combined abiotic and biotic stressors. In fact, combined effect of ecological and chemical aspects is a hot topic in ecotoxicology nowadays (De Laender *et al.,* 2013; Seeland *et al.,* 2013; De Coninck *et al.,* 2013). European Union supports the relevance of this topic financing projects as "Novel Methods for Integrated Risk Assessment of Cumulative stressors in Europe (NoMiracle)". The main objective of NoMiracle is to better understand complex exposures including mixture of chemicals and physical/biological factors in risk assessment what is in line with these thesis objectives.

In the **previous five chapters**, microcosms experiments were set up. Microcosms studies could be one appropriated experimental scale to assess complex pressures scenarios representing a compromise between uncertainty and ecological realisms. In order to face uncertainty, a strategy could be the use of multiple scales experiments. The combination of different scales and complexity experiments can help to understand the lower mechanisms controlling the higher scales responses. For instance, a shift in community structure observed in microcosms experiments could be driving for food competition pressures affecting predation/cannibalism behaviours that can be recorded at lower scale experiments of two interacting species. This was the conceptual framework to set up the experiment in **chapter 6**. In **that chapter**, effects of intra- and interspecific competition were assessed on the sensitivity of aquatic macroinvertebrates to a fungicide (carbendazim). As expected, the results were complex showing how competition influences the response of organisms under chemical exposure depending on diverse factors asspecie, density and behavioral aspects. In the experiment with *Bithynia tentaculata* and *Radix peregra, B. tentaculata* growth was affected by combined effect of competition and carbendazim; however, survival and immobility were only transiently affected. The adverse effect detected on growth rear concern about fertility and new generation fitness to face chemical exposures. In the experiment with *Gammarus pulex* and *Asellus aquaticus* a surprisingly positive influence of competition was observed what is explained by behavioral changes (cannibalism and predation) able to modulate toxic effects under specific combinations of competition and chemical pressures. It points out the complexity of organisms responses under combined abiotic and biotic pressures, therefore, the challenging extrapolation of results to higher scales as community and ecosystems. Consequently, these experimental results support the need of more ecological realistic risk assessment. And, it contributes to the literature identifying diverse organisms' responses to combined pressures: the negative competition influence detected that agree with previous studies; and, the unexpected positive competition influence what open a new discussion window.

4. Science-based decisions: scientists tools useful for decisions makers

Science has the responsibility to transfer knowledge to society. The main barriers for this knowledge transfer are results interpretation and implementation. Therefore, apart from accurate environmental and ecological experimental designs; the way in which data are presented is extremely important.

This thesis claims the need of more realistic scenarios for ecological risks assessments together with the need of appropriated tools to make that knowledge socially relevant. Hence, comprehensible knowledge will trigger managers and social awareness that is the engine of policy changes as could be the procedure to establish legal limits. Under this framework, Principal Response Curves (PRC) was used in all experiments because they could be a tool combining science accuracy and friendly interpretation for no-scientist audiences. PRC is considered scientifically a more appropriated tool than analysis of variance to analyzed micro/mesocosms data. PRC couples complex changes in community structure and dynamics over time as a result of environmental changes (i.e., agrochemical exposures). Therefore, it adds the time dimension to the data analysis considering community dynamics what sum up to community structure information gives a global and integrate picture of the system resulting in a realistic ecological scenario. In addition, at social levels it could be a friendly method for data presentation to engage a general audience in a more based-science decisions owing to its visual and intuitive interpretation.

5. Ecological meaning of experimental results on field conditions

In accordance with the efforts to use scientist tools useful for policy makers, an attempt to translate experiment results into relevant field information has been also made. In order to do so, the Predicted No-Effect Concentration (PNEC) has been calculated with some modifications. The PNEC is calculated using the quotient method comparing toxicity to environmental exposure by the relation of estimated environmental concentrations (EEC) and an effect level such as the LC50 based on single species test endpoints (survival, growth and reproduction). Toxic risks do not exists when the quotient is 1 or below; however, toxic risks exists when the quotient is higher than 1. The modification introduced here is to use the lower concentrations that trigger adverse effects at microcosm levels (it will be defined as Community Negative Effect Concentrations, CNEC, figure 1a) instead of the LC50. It aims to overcome the shortcomings of using LC50 from endpoints based on single species tests and its low environmental realistic experimental conditions. Hence, it pretends to use the lowest concentrations having effects on microcosms which experimental design are able to include diverse ecological (populations and community) and chemical scenarios (mixtures and frequency). Therefore, it is assumed that the information will be more realistic for make better field risk predictions. The PNEC will be calculated for Andalucía region where this thesis impact is more relevant and field agrochemical data are available (Table I). Therefore, nutrients and pesticides concentrations data in wetlands are taken from the Junta de Andalucía monitoring program from 2002 until 2007 (Junta de Andalucia, 2002-2007). In addition, Robles-Molina *et al.* (2014) published data were considered for its higher detection limits of Chlorpyrifos. Carbendazim data were not available (Table I). We have decided to classify the PNEC risk values in three categories: no risk, risk and extreme risk (Figure 1b). It is considered “no risk” between 0 – 1 due to the own PNEC method defition; “risk” any value higher than 1 because it means that the exposure will have effects on the systems; and, “extreme risk” when it is an order of magnitude higher than the minimum risk scenario.

a)

PNEC = (EEC / CNEC)

b)

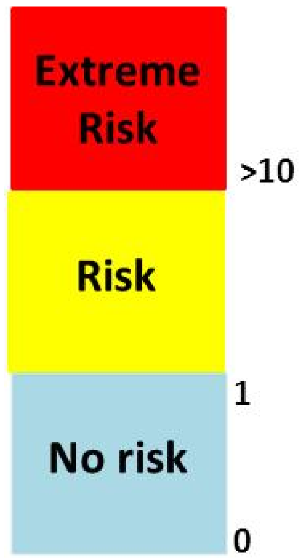

Figure 1.a) Quotient to calculate the Modified Predicted No-Effect Concentration (PNEC).
b) Risk categories

Table I. Results of the Predicted No-Effect Concentration (PNEC) based on field concentrations monitored by the Junta de Andalucía (Estimated Environmental concentrations, EEC) and this thesis microcosms experiment results (Community Negative Effect Concentrations, CNEC are in bold being the lowest concentrations having negative effects). Legal limits data are also included.

Agrochemical	CNEC	EEC	Legal limits	PNEC
Copper	0.2 mg l^{-1} **0.02 mg l^{-1}** 0.002 mg l^{-1} 0.04 mg l^{-1}	* 0.06 mg l^{-1}	0.04 mg l^{-1}	Risk (3)
Nitrate	**25 mg** l^{-1} 50 mg l^{-1}	1.98 mg l^{-1}	50 mg l^{-1}	No risk (0.1)
Chlorpyrifos	**0.002 mg** l^{-1}	**< 0.001 mg l^{-1} (1.38 *10^{-5} mg l^{-1})	0.0001 mg l^{-1}	No risk (0.1)
Carbendazim	0.4 mg l^{-1} 0.8 mg l^{-1} 1.2 mg l^{-1}	n.a.	n.a.	n.a.

*Above legal limits **It is considered that more accuracy is needed; therefore, the worst case scenario of 1.38 x 10^{-5} mg l^{-1} from Robles-Molina *et al.* (2014) was used as EEC. n. a.: stands for not available

Monitored field data from the Junta de Andalucía compare to legal limits and the results of the PNEC revels that Andalucian wetlands are under risk of impairment for copper and no risk for nitrate and chlorpyrifos. Carbendazim data were not available in the Junta de Andalucía monitored program and was not detected by Robles-Molina *et al.* (2014). Field concentrations of copper are above legal limits; while, nitrates and chlorpyrifos are within legal limits based on the average monitored concentrations. And, the PNEC based on those average monitored field concentrations (EEC) and the Community Negative Effects Concentrations (CNE) from the microcosm's experiments results on risk for copper and no risk for nitrates and chlorpyrifos. At first glance, copper seems the only priority issue. However, a precautionary aptitude raises some questions about the no risk of nitrates and chlorpyrifos. In the case of copper and nitrates, the average of the estimated environmental concentrations marks some concentrations peaks (Junta de Andalucía, 2002-2007). Nitrates peaks average is 37.6 mg l^{-1} and the range (25.6 – 51.3) mg l^{-1}, peaks mainly occurs on November and December.Olive groves are the main agricultural activity in our region, fertilizers are applied on autumn therefore peaks of nitrates on November and December could be linked to fertilizers mobilization by raining events. In the case of copper, peaks average is 0.122 mg l^{-1} and the range (0.05 – 0.46) mg l^{-1}, peaks mainly occurs on April and November. Copper sulfate is used as a fungicide and is applied before the most humid months; therefore, peaks on April and November are consistent with such field application timing. It highlights the important of consider frequency aspect when studying agrochemical exposures scenarios as it has been pointed out in chapter 5. With respect to chlorpyrifos, its concentrations were lower than 1 µg l^{-1} (Junta de Andalucía, 2002-2007), however, it seems that the detection limit may not be sensitive enough. Robles-Molina *et al.* (2014) monitored Jaén wetlands up to nanograms (ng l^{-1}) and reported the present of chlorpyrifos in 73% of the samples even having one of them slightly above (119 ng l^{-1}, Guadalimar river) legal limits (100 ng l^{-1}). In conclusion, the no risk detected based on the data available could be underweight owing to monitoring limitations. Monitored agrochemical concentrations is crucial to obtain realistic information about the importance of scales, spacial and temporal aspect of agrochemical pulses what is essential to better understand ecological consequences. For instance, Hanazato (1998) highlighted the consequences of zooplankton abundance and structure changes as a consequence of insecticides exposures on winter having effects on the spring communities by altering the clear-water phase and fish larvae development. Different insecticide timing exposures could have different consequences modulated by which species are present and its development stage, among other biotic and abiotic factors.

6. Transfer to society: recommendations

Society should seek to find the balance between agriculture development and environmental protection despite of its complexity. It should be notice that food supply is one of the ecosystem services society obtain from healthy ecosystems (EEM, 2012). Therefore, efforts to prevent ecological impacts come together with the need of agriculture. Simple but significant recommendations are enlisted below:

- ✓ "Prevention is better than cure". Therefore, **a decrease of pesticide use** will have economic and environmental benefits because it will decrease the expenses in the agricultural sector and reduce environmental risk saving restoration investments. It comes through a careful planning of agrochemical needs.

- ✓ **Prevention of runoff**. The most common entrance of agrochemicals in aquatic systems is through runoff. Therefore, apart from the use of adequate agrochemical concentrations also application techniques diminishing runoff probability should be consider: injection of pesticides, increase of vegetation around water bodies or establishment of buffer zones between application areas and aquatic ecosystems.

- ✓ Investments on alternative agricultural practices as **ecological agriculture**. Enhance ecological agriculture could be an important strategy to solve the problem at its root cause. However, the safety of natural compounds used in ecological agriculture must be scientifically proven and not assumed.

- ✓ Scientific results must be relevant, credible and legitimate for society in order to **involve society** in its application. **Experiments designs with environmental realisms to target social issues** are the key to implicate audiences to follow up with actions.

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CONCLUSIONS

1. Copper current legal limits are not safe enough because impacts on aquatic community were detected and high environmental risk was calculated. Plankton community responses to environmentally relevant agrochemical concentrations within legal limits warn about adverse effect at structural levels. Even if such effects are transient, they raise concern about long term effects on plankton communities.

2. Mixture of agrochemical exposures allows understanding how agrochemical interactions modulate indirect effects resulting in different community responses than those showed in single agrochemical exposures. Therefore, agrochemical mixture studies have been configured as essential in ecotoxicology.

3. Phytoplankton abundance endpoint is essential to detect both direct and indirect effects of agrochemicals on the community. Direct effects show a decrease of phytoplankton abundance owing to a clear toxic effect on the species. While, indirect effects, leading to abundance decrease or increase, give information mainly related to changes of zooplankton grazing pressure resulting from its depletion or community shifts after agrochemical exposures. In addition, generally phytoplankton community response was faster than zooplankton one most likely owing to a shorter life cycle.

4. In terms of phytoplankton community structure based on cytometry analysis of cell size changes, no conclusive results were obtained. Probably phytoplankton sampling procedure must have been adapted to its shorter life cycle; therefore, weekly sampling was not the most appropriate timing to detect community structural changes.

5. Zooplankton community structure gave more valuable information than phytoplankton abundance changes. Zooplankton taxa shifts are better indicators because it allows understanding zooplankton community responses and phytoplankton indirect effects.

6. Functional endpoints (oxygen production and litter decomposition) did not help to identify agrochemicals' effects on plankton community. Sampling procedure and methodology should be review and adapted to microcosms design and experiment duration.

7. Microcosms community studies allow interpreting complex responses mediated by indirect effects and compensatory mechanisms. Considering this, two species competition experiments are an adequate scale to study combined effect of ecological and chemical aspects, which results may help to interpret responses at microcosm's community levels. The influence of food availability (competition) on organisms and community responses to agrochemical exposure has been confirmed. Therefore, competition as other ecological interactions arises as an essential factor to be taken into account to increase the ecological realisms of risk assessment.

CONCLUSIONES

1. Los límites legales de cobre actuales no son suficientemente seguros para la comunidad acuática puesto que se han detectado impactos negativos y se ha calculado un alto riesgo ambiental. Las respuestas de la comunidad planctónica a concentraciones de agroquímicos medioambientalmente relevantes y dentro de los límites legales muestran efectos a niveles estructurales. A pesar de que dichos efectos pueden ser pasajeros, no deben despreciarse al considerar los efectos a largo plazo y su repercusión.

2. La exposición a mezclas de agroquímicos ha permitido entender como dichas mezclas modulan los efectos indirectos que resultan de las respuestas de las comunidades expuesta y que difieren de las respuestas a la exposición de un único agroquímico. Por tanto, las mezclas de agroquímicos se destacan como un punto esencial en el incremento de realismo de los estudios de ecotoxicología.

3. La abundancia de fitoplancton como indicador de punto final o endpoint es esencial para detectar tanto efectos directos como indirectos de los agroquímicos sobre la comunidad planctónica. El descenso de la abundancia de fitoplancton es un claro efecto directo de los tóxicos sobre las especies. Mientras que los efectos indirectos ligados a un aumento o descenso de la abundancia, dan información principalmente relacionada con los cambios en la comunidad tras la exposición al tóxico como reflejo del cambio en la presión de herbivoría del zooplancton debido a su declive o a los cambios en la composición del mismo . Además, en

general la respuesta de la comunidad fitoplanctónica fue más rápida que la del zooplancton debido al ciclo de vida más corto.

4. No se han obtenido resultados concluyentes cuando se han analizado los cambios en la estructura de la comunidad fitoplanctónica basada en análisis del tamaño celular mediante citometría de flujo. Probablemente la metodología de muestreo del fitoplancton en las experiencias de microcosmos debería haber sido adaptada a su corto ciclo de vida; los muestreos semanales no han sido periodos adecuados para detectar los cambios estructurales basados en el tamaño celular en la comunidad.

5. La estructura de la comunidad zooplanctónica proporcionó una información más valiosa que los cambios de abundancia del fitoplancton. Los cambios en taxones del zooplancton son mejores indicadores porque permiten entender las respuestas de la comunidad zooplanctónica y los efectos indirectos sobre el fitoplancton, relacionándolos con interacciones ecológicas.

6. Los indicadores funcionales (la producción de oxígeno y la descomposición de hojarasca) no ayudaron a identificar los efectos de los agroquímicos sobre la comunidad planctónica. El procedimiento de muestreo y la metodología deberían ser revisados y adaptados a diseños con microcosmos y a la duración del experimento.

7. Los estudios de microcosmos de la comunidad acuática permiten interpretar respuestas complejas mediadas por efectos indirectos y mecanismos de compensación. Tomando esto en consideración, los experimentos con dos

especies son una escala adecuada para estudiar los efectos combinados de aspectos ecológicos y químicos, cuyos resultados podrían ayudar a interpretar respuestas a niveles superiores de complejidad. La influencia de la disponibilidad de recursos (competencia) en la respuesta de los organismos y de la comunidad a la exposición de agroquímicos ha sido confirmada. Por tanto, la competencia, así como otras relaciones ecológicas, surgen como un factor esencial a ser considerado para aumentar el realismo ecológico en la evaluación del riesgo de los agroquímicos.